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ENZYME-LIKE GLOBULINS FROM SERUM REPRODUCING THE VASCULAR PHENOMENA OF INFLAMMATION

V. ACTIVABLE PERMEABILITY FACTOR IN HUMAN SERUM

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THE main globulin permeability factor (PF) of the blood in guinea-pig (Miles and Wilhelm, 1955; Wilhelm, Miles and Mackay, 1955) and rat (Wilhelm, Mill, Sparrow, Mackay and Miles, 1958) exists as an inactive precursor (pro-PF), and an inhibitor (IPF) of the PF is present as such. The conversion of pro-PF to PF, and the subsequent inhibition of the activated PF, is evident when serial dilutions of fresh guinea-pig or rat serum in 0·85 per cent saline are tested for PF potency, at varying intervals after dilution. The two outstanding "dilution-effects", as seen in guinea-pig serum, are (*a*) the appearance after 1 hr. of a zone of maximum PF activity in the dilution range 1/135–1/405, and the absence of activity in the range 1/1 to 1/50, owing to the inactivation by IPF in these lower dilutions; and (*b*) the disappearance after 4 days of PF activity from the 1/135 dilution, owing to the progressive action of the low concentration of IPF, and the appearance of PF in the previously inactive range 1/1215–1/3645 (Wilhelm, Mill and Miles, 1957). Dilution effects of this kind, in fact, are useful indicators of the presence of a pro-PF/IPF system in biological fluids (Miles and Wilhelm, 1958), including the serum of animals hitherto unexplored in this respect. The dilution effect cannot, for example, be elicited in rabbit serum (Wilhelm *et al.*, 1958) but, as we record in this paper, it is demonstrable in human serum.

As in our previous work, PF potency was measured in terms of local exudation into intracutaneous injection-sites of a blue dye present in the animal's circulation. For obvious reasons, it was impracticable to apply the principle that has hitherto governed the choice of animal for investigation, and to make any extensive analysis of the action of the various serum permeability factors in the homologous animal, namely man. The guinea-pig was used as the test animal; it was not therefore

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possible to distinguish permeability effects that would also be elicited in human tissue from those due to some species incompatibility between guinea-pig and man. Our results nevertheless indicate the presence in human serum of a pro-PF readily activated by simple dilution, confirming the observation of Stewart and Bliss (1957), and suggest the presence of an IPF.

MATERIALS AND METHODS

Blood.—Human blood was collected by venepuncture from healthy adults, defibrinated and centrifuged at 4000 r.p.m. for 5–10 min.; the serum was stored at 4°. Unless otherwise stated, dilutions were made in 0·85 per cent sterile saline.

PF potency.—Permeability-increasing potency, its diminution by various inhibitors and the time-course of the permeability effect were measured in terms of lesion-diameters in the skin of depilated, " blued " albino guinea-pigs—*i.e.*, animals with pontamine blue in their circulation—as detailed by Miles and Wilhelm (1955). The standard injection-volume was 0·1 ml., and in any one experiment, mean diameters were derived from 4–16 replicate injections of each test preparation, made in 4 guinea-pigs. The " effective blueing dose " (EBD) of a permeability factor is defined as the amount in 0·1 ml. inducing on the average a lesion-diameter of 6 mm. at the time of maximum development of the area of increased vascular permeability (see Wilhelm *et al.*, 1958). In the guinea-pig, the lesion-diameter was estimated 30 min. after the intracutaneous injections.

RESULTS

A Low-potency, Native PF (PF/Nat) in Undiluted Human Serum

Unlike fresh guinea-pig and rat serum, but like fresh rabbit serum, undiluted human serum contains an active PF (PF/Nat). Within 3 min. of injection into the blued guinea-pig it induces a moderately intense blue lesion, 12–14 mm. in diameter (Fig. 1), the centre of which usually becomes haemorrhagic after 5–10 min. The effect is not antagonized by high concentrations of soya bean trypsin inhibitor (SBTI) 200 μg./ml., or of guinea-pig IPF (fraction G1S/P) 1·65 mg./ml. In this it is distinct from the main PF of guinea-pig, rat and (see below) human serum, and it is not affected by mepyramine maleate, 20 mg./kg. given intraperitoneally to the guinea-pig 2 hr. before the intracutaneous test. It resembles, therefore, the native PF that appears in rat serum more than 1 hr. old (PF/Nat) and like rat PF/Nat, may result from a rapid *in vitro* degenerative change in the serum ; a degeneration analogous with the much slower formation of PF/Age in guinea-pig serum held for 1–4 days at 4°. One ml. serum contains some 100 EBD of PF/Nat.

Human PF/Nat is further distinguished from the main PF in all mammalian sera so far tested, by its time-course. Whereas the increased capillary permeability induced by the latter lasts for 15–20 min., that induced by PF/Nat lasts more than 1 hr. PF/Nat is relatively heat-labile, a 1/5 dilution losing all its potency after 30 min. at 65° (see Fig. 4).

The dilution effect

The ageing of serum dilutions for 1 hr. at room temperature, which reveals a peak of PF activity in high dilutions of guinea-pig and rat serum, fails to elicit a similar response in dilutions of human serum. A peak of activity appears after more prolonged ageing. Fig. 1 summarizes the changing activity of a dilution series ; the dilutions aged 10 min. and 2 hr. (curves A and B) were held at room

temperature ; those aged 1, 2 and 4 days (curves C, D and E) were held for 10 min. at room temperature and then at 4°. Ten minutes after dilution, the curve of activity is maximum in undiluted serum and declines at about 1/100 to the base-line of traumatic " blueing " due to needle-injury. The slope of the decline, against log. dilution of serum, is about 4·5, which is consistent with the simple dilution-out of the PF/Nat present in undiluted serum. At 2–6 hr., however, the slope is interrupted by a short flat portion in 1/25–1/75 serum, and has a value of about 2·6 over the whole range of dilutions, suggesting the addition of an effect due to a second PF developing in low dilutions during this period. After 1 day,

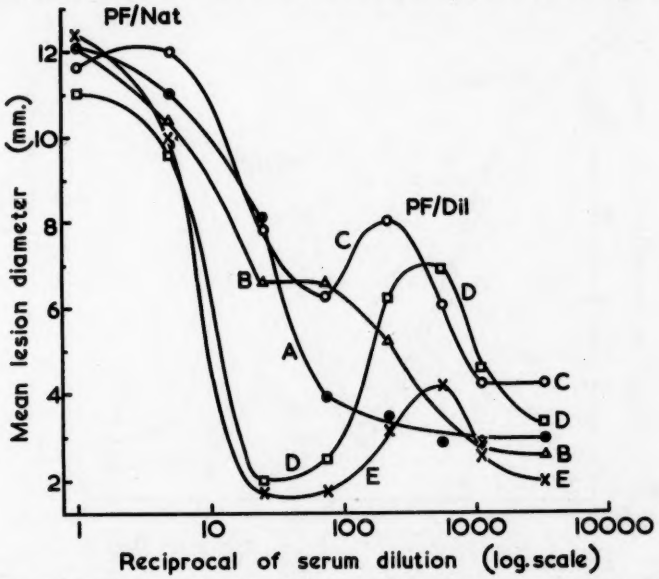

FIG. 1.—The progressive activation of PF/Dil in serial dilutions of serum after intervals of 10 min. (A) and 2 hr. (B) at room temperature, and of 1 day (C), 2 days (D) and 4 days (E) at 4°.

a peak appears at 1/225, and moves after 2 days to 1/550. During the same period, the trough at 1/75 in the 1-day curve deepens, and after 4 days, the 1/25 and 1/75 dilutions are inactive, and the slope of the presumed dilution-out of PF/Nat is as steep as 6·5.

By analogy with sera of other species, the peak is due to the activation of a PF/Dil, and the trough to the progressive inactivation of the PF/Dil by a slowly acting IPF, which does not affect the PF/Nat. The curves in Fig. 1, however, offer no evidence of a progressive activation of PF/Dil after 1 day, such as occurs in dilutions of guinea-pig serum (Wilhelm *et al.*, 1957). The rightward shift of the PF peak at 2 days can be explained solely in terms of an encroachment of the IPF trough on a stationary peak of PF/Dil activation ; and at 4 days there is a general decline in the peak, partly attributable to IPF encroachment, but perhaps also to an instability of PF/Dil itself (see below).

The trough of IPF inhibition forms more quickly at higher temperatures. The curves in Fig. 2 illustrate what proved to be one of the best methods for eliciting a good dilution effect, as revealed in guinea-pig skin, namely activation of a dilution series for 44 hr. at 4°, followed by 1 hr. at 37°. Curve B is the response commonly obtained, whereas curve A is an exceptionally high PF/Dil peak. It is evident from curve A that in optimum conditions of activation 1 EBD (≡ 6·0 mm. lesion) is activated at a dilution of 1/500. One ml. of undiluted serum therefore contains about 5000 EBD of activable PF/Dil.

The dilution effect is similar when mammalian Ringer, Locke's solution, or phosphate buffer pH 8·0, I = 0·2, are substituted for 0·85 per cent saline. This

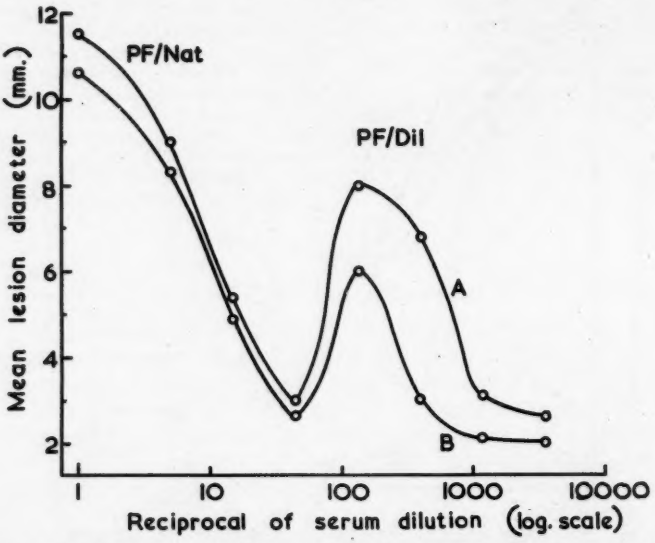

FIG. 2.—The optimal activation of PF/Dil in serial dilutions of serum, held for 44 hr. at 4° and 1 hr. at 37°. Curve A represents an exceptionally good activation, curve B the more usual activation.

method of eliciting a dilution curve with a pronounced trough between the PF/Nat and PF/Dil zones is also effective with undiluted serum held for 7–21 days at 4°. Compared with that in guinea-pig serum (Miles and Wilhelm, 1955), the activation is less striking. At the peak, the lesion-diameter is usually not greater than 7 mm. and the intensity of blueing not more than ++.

Lewis and Work (1956) cited the activation of plasmin in diluted human plasma (Macfarlane and Pilling, 1946; cf. Dausset, Bergerot-Blondel and Colin, 1956) as a phenomenon resembling the activation by dilution of a substance in human serum stimulating smooth muscle. The activation of PF and of plasmin by dilution of human serum might also be expressions of the same kind of process. The last two effects are not however analogous. The PF dilution effect occurs with *normal* sera, whether human, rat or guinea-pig. Macfarlane and Pilling simply unmasked plasmin that had already been activated *in vivo*, by diluting out the

native plasmin-inhibitor present in the plasma : the normal plasma did not display any such effect.

Properties of PF/Dil

Stability to storage.—The activated PF/Dil in high serum dilutions becomes inactive after 1 day at room temperature and after 4 days at 4°. It remains active for at least 3 weeks at — 10°.

Stability to heat.—Activated PF/Dil is largely destroyed after heating to 65° for 30 min. (Fig. 4).

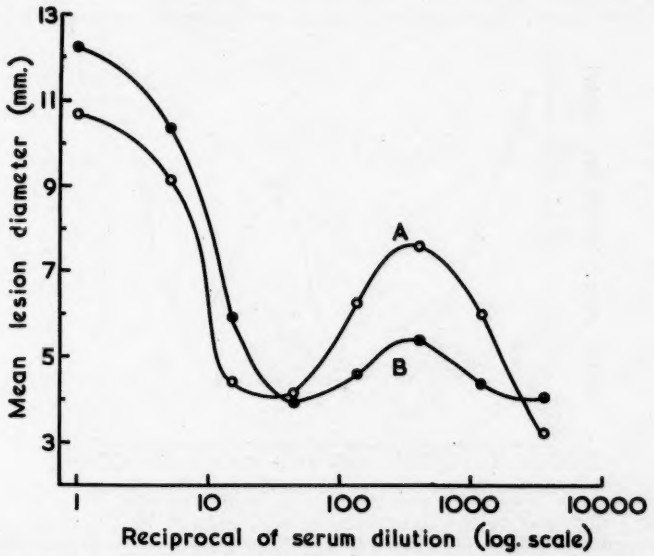

FIG. 3.—The partial suppression of the activation of PF/Dil in human plasma from blood treated with heparin on withdrawal (curve B). The serum activated to provide the control (curve A) was obtained from the same sample of blood, defibrinated on withdrawal.

Time-course.—The duration of increased capillary permeability induced by PF/Dil was measured with the 1/405 dilution, at the peak of activation. The PF effect was short, permeability returning to its normal low level within 5 min. This is a shorter time-course than that of guinea-pig PF/Dil where the time-course is 15–20 min. The shortness however is probably attributable to the small amount of PF activated in the optimum dilution, and not to any peculiarity of human PF/Dil ; because many other permeability factors, including histamine and serum PF from other species, with a time-course of 15–20 min., have a much shorter time-course when used in minimally effective concentrations.

Susceptibility to inhibitors.—Human PF/Dil is highly susceptible to SBTI, which has a pronounced effect in concentrations as low as 0·5 μg./ml. Concentrations of SBTI, 10 μg./ml., and of the guinea-pig serum fraction G1S/P, 1·65 mg./ml., which is rich in IPF, both remove all activity from maximally activated

preparations of PF/Dil, after 30 min. at 37°. Intraperitoneal mepyramine maleate, 20 mg./kg., 2 hr. before the intracutaneous assay—a dose that suppresses the response to histamine, 10 μg./ml. (Miles and Miles, 1952)—failed to affect the PF/Dil.

In its susceptibility to SBTI and guinea-pig IPF and insusceptibility to mepyramine, human PF/Dil closely resembles the PF/Dil of guinea-pig serum.

Modification of the dilution effect

By ageing of serum.—The pro-PF in undiluted serum is moderately stable. It can be activated in serum held at room temperature for 1 day, but has disappeared after 4 days. Undiluted serum held at 4° is readily activable after 4 weeks,

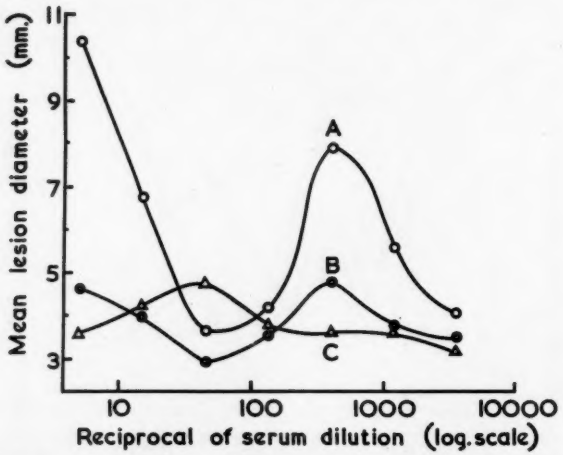

FIG. 4.—The lability of PF/Nat and PF/Dil on heating for 30 min. at 65°. A = Serum dilutions activated for 44 hr. at 4° and 1 hr. at 37°. B = Serum dilutions activated as in A, and then heated. C = Serum dilutions heated, and then activated as in A.

but not after 6 weeks; during the first week, the activability improves, and remains high until the third week. At −10°, serum retains its activability for at least 4 weeks.

By heparin.—The dilution effect of heparinized plasma (heparin, 2 mg./ml. blood harvested) differs from that of serum; there is (Fig. 3) a slight enhancement of the PF/Nat curve, and a substantial suppression of the PF/Dil peak, amounting to a 75 per cent reduction of potency in the maximally activated dilution. The addition of heparin to already activated serum dilutions, on the other hand, does not affect the PF/Dil peak. The suppression of the dilution effect closely resembles that produced in guinea-pig serum by the anticoagulant dextran sulphate (Wilhelm *et al.*, 1957). The pro-PF/IPF system in human serum, however, appears to be more sensitive to suppression, because heparin in moderate anticoagulant concentrations does not modify the dilution effect in guinea-pig serum.

By heat.—PF/Dil is activable in undiluted serum held for 30 min. at 55°, but not to any substantial extent in serum heated for 30 min. at 65° (Fig. 4).

By dialysis.—Prolonged dialysis of serum dilutions removes the trough of inhibition in the range 1/45–1/135, and indeed enhances the PF activity of all dilutions from 1/5–1/1215 (Fig. 5). The enhanced activity displayed in all parts of the dilution curve is presumably due to PF/Dil, because it is susceptible to both SBTI and guinea-pig IPF. The SBTI removes all activity of the dilutions beyond 1/135; in low dilutions of serum, it leaves unchanged an activity corresponding to that of the original PF/Nat. Dialysis therefore appears to inhibit the mechanism whereby the PF/Dil activated in low dilutions is suppressed by the native inhibitor, IPF, suggesting that a dialysable substance is essential for the action of IPF. A similar effect of dialysis on the dilution effect was also

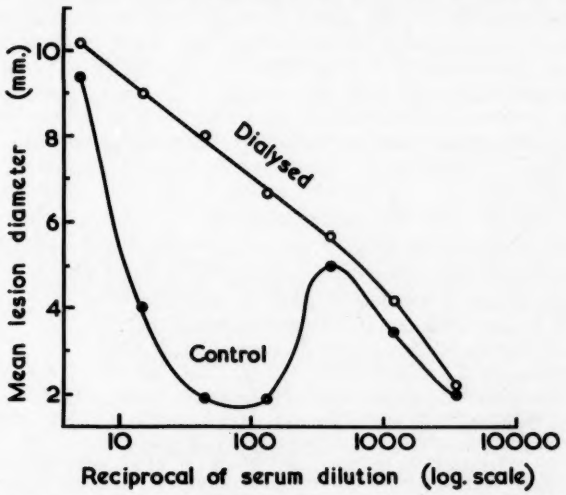

FIG. 5.—The effect of dialysis against 0·85 per cent saline for 3 days at 4° on the PF activity of serial dilutions of human serum.

observed in guinea-pig serum (Wilhelm *et al.*, 1957); and in that species it was not possible to demonstrate the removal of a diffusible factor necessary for inhibition. The phenomenon is under investigation.

SUMMARY

Dilutions of human serum in saline, tested in the skin of the guinea-pig for their effect on the permeability of the small blood vessels, contain two permeability factors (PF).

One, PF/Nat, is native to freshly harvested, undiluted serum and usually is diluted-out at 1/45. It is insusceptible to inhibition by soya bean trypsin inhibitor (SBTI), by IPF, the PF-inhibitor of guinea-pig serum, and by the antihistamine drug, mepyramine maleate. PF/Nat presumably corresponds to the permeability factor in undiluted human serum described by Boake and Lovell (1954), and to the PF/Age that appears in low dilutions of ageing guinea-pig serum.

The second, PF/Dil, is activated from an inert pro-PF by dilution and is detectable 2–6 hr. after dilution of serum to 1/50, and after 1–4 days in the range 1/100–1/1000. In the older dilutions, a zone of inhibition separates the zones of activity due to PF/Nat and PF/Dil, which, by analogy with a similar phenomenon in diluted guinea-pig serum, appears to be due to the slow inhibition of PF/Dil by an inhibitor (IPF) present in the serum. Human PF/Dil, like guinea-pig PF/Dil, is inhibited by SBTI and by guinea-pig serum IPF, but not by mepyramine.

The dilution effect with human serum as manifested in the skin of the guinea-pig is almost certainly analogous with the dilution effect demonstrated in the skin of man by Stewart and Bliss (1957), and the PF/Dil identical in the two cases. These authors found no PF in undiluted serum; it appears that the potent PF/Nat we found by guinea-pig assay owes its activity to a species incompatibility between a component of human serum and the tissues of the guinea-pig. The discrepancy illustrates the importance, whenever it is possible, of testing endogenous permeability factors in the tissues of the homologous animal.

We are indebted to the Nuffield Trust for its financial help in the prosecution of this work.

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ENZYME-LIKE GLOBULINS FROM SERUM REPRODUCING THE VASCULAR PHENOMENA OF INFLAMMATION

VI. ISOLATION AND PROPERTIES OF PERMEABILITY FACTOR AND ITS INHIBITOR IN HUMAN PLASMA

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IN this paper we record the partial purification and characterization of human serum-globulin permeability factor (PF) corresponding to the PF/Dil activable in human serum by simple dilution (Stewart and Bliss, 1957; Elder and Wilhelm, 1958) and of a protein inhibitor (IPF) of that factor. The PF differed from the analogous factors in the sera of the guinea-pig and the rat in being associated with the β- rather than the α-globulins—in this respect partly resembling the serum PF of the rabbit, which is associated with both α- and β-globulins—and from the serum PF of all three animals in its relative instability.

MATERIALS AND METHODS

Blood.—One batch of serum was investigated but otherwise citrated plasma (120 ml. 3 per cent $Na_3C_6H_5O_7$. $2H_2O$ to 420 ml. blood) from the blood of young adult donors, harvested as for transfusion, was used.

Assays.—The properties of the PF and the IPF were determined by methods previously described for guinea-pig serum (Wilhelm, Miles and Mackay, 1955; Wilhelm, Mill and Miles, 1957) using the guinea-pig with pontamine blue in its circulation as the test animal. The measure of potency of substances which induce linear and parallel dosage-responses in this system is the effective blueing dose (EBD); this is the quantity of permeability factor, in 0·1 ml. saline, which when injected intracutaneously into the blued guinea-pig induces on the average a lesion 6 mm. in diameter at the time of the maximum development of the area of increased permeability (Wilhelm *et al.*, 1958). In the guinea-pig, lesion-diameter was estimated 30 min. after the intracutaneous injections. To facilitate the comparison of the results from different assays injections of a standard preparation of guinea-pig serum PF (G2/1R, Batch 33) were incorporated into the test system. This preparation has a potency of 10,000–20,000 EBD/mg. protein when tested in the laboratory strain of guinea-pig and the potency of other factors is expressed in terms of this guinea-pig standard. The potency ratio of the unknown to the standard was measured at the 6 mm. level from dosage-response lines fitted by eye to the mean lesion-diameters obtained in the assay. These mean lesion-diameters were estimated from at least 4 lesions, each in a different animal.

Fractionation.—Ether fractionation of serum protein by the standard method of Kekwick and Mackay (1954) yielded preparations of which G2/1, consisting mainly of α- and β-globulins, was the most potent PF. Its potency however was low and higher potency preparations were obtained by the method described below.

Plasmin activity.—Plasmin was measured by caseinolysis (Wilhelm *et al.*, 1958), and plasmin proactivator by the lysis of bovine fibrin clot in the presence of excess streptokinase and

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plasminogen (Mill, 1957). The blood plasminogen activator—" spontane " activator—was estimated by the heated fibrin-plate technique (Lassen, 1952).

Electrophoresis.—Paper electrophoresis on a free horizontal paper (Whatman's 3 MM) was carried out in a sealed container using phosphate buffer pH 8·0, I = 0·15. The strips were developed and the colour measured by the method of Kunkel and Tiselius (1951).

RESULTS

Human PF Activated During Serum Fractionation

The G2/1 fractions of three batches of " time-expired plasma " (*i.e.*, at least 4 weeks old) were prepared by the standard method; and had about 1/150th of the PF potency of the standard guinea-pig PF. The low potency may have been

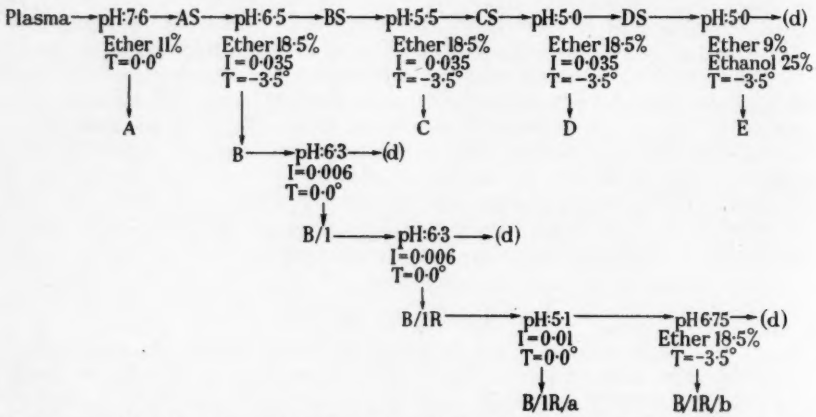

FIG. 1.—Flow sheet for the separation from human plasma of the potent PF fraction, B/1R a, and of the fraction D, containing 1PF. Horizontal lines indicate supernatant solutions; vertical lines indicate precipitates.

T = temp., I = ionic strength, (d) = discoid.

due in part to incomplete activation of PF from pro-PF and to failure to separate PF and IPF, because on one occasion a sub-fraction G2/2, like fresh serum was activated by dilution (Elder and Wilhelm, 1958); a freshly prepared solution of this G2/2, 500 μg./ml., was inactive when injected immediately but contained about 2 EBD after standing 3½ hr. at room temperature.

The preferred method of fractionation (Fig. 1) was devised after tests in which the age of the plasma on fractionation and the pH, ionic strength and solvent concentration at successive stages were treated as independent variables. One feature of the preliminary investigation is noteworthy. Elder and Wilhelm (1958) observed that storage of undiluted serum for about a week at 4° improved the degree to which PF was activated on dilution. A similar improvement in the yield of active PF in the crude fraction B was observed when the first supernatant S1 of the Kekwick and Mackay scheme was allowed to age at 2°. After 7 days the S1 fraction yielded 6–8 times more PF than fresh S1. But fresh S1 yielded more potent PF fractions than old S1 when the fractionation process was carried to the

B/1R/a stage described in the next paragraph; presumably because further activation of pro-PF took place during the later stages of fractionation. The method is as follows.

The citrated plasma from 5–6 donors is collected by centrifugation, cooled as rapidly as possible to 0° and pooled. Fractions A to E are successively precipitated at the pH, ionic strengths, solvent concentrations and temperatures shown. Fraction B is further purified to give B/1R, which is then split into the potent PF, B/1R/a, and the inactive B/1R/b. This separation is facilitated by replacing the phosphate-acetate buffers recommended by Kekwick and Mackay with calcium acetate buffers. The inhibitor IPF (see below) is in fraction D. For all precipitations, excepting stage A, the mixtures are allowed to equilibrate overnight. For the best separation of PF and IPF it is of first importance that the plasma is not more than 2–3 hr. old, that the precipitate A is removed 1 hr. after adding the ether and that the precipitation of B from AS is begun immediately.

The results of a typical fractionation are summarized in Table I. Fraction A, which is discarded, is largely fibrinogen and constitutes about 10 per cent of the total plasma proteins. Because of the difficulties of handling such fractions, with their high fibrin content, the analysis in Table I is presented in terms of the protein content of AS. The greater part of the PF is activated in the step B → B/1R, the potency rising 10-fold (320/30) with a loss only of one-quarter of the protein. A little more PF is perhaps activated during the separation of B/1R/a and B/1R/b, the total EBD in the two being about 25 per cent more than that in B/1R, in spite of a loss of 20 per cent of the protein in the process; and the bulk of this activity is in B/1R/a, which has 3 times the potency of B/1R. The PF potency of fractions C, D and E is low, though, owing to its large amount, fraction E accounts for one quarter of the total PF.

TABLE I.—*A Typical Fractionation of the AS Fraction of* 1700 *ml. Human Plasma*

Fraction	Yield		PF potency	
	Total protein (mg. N × 6·25)	Percentage of AS protein	EBD per mg.	EBD × 10^{-3} per fraction
AS	71,000	100	—	—
B	9,250	13	30	278
B/1R	7,100	10	320	2,272
B/1R/a	2,830	4	1,000	2,830
B/1R/b	2,840	3	22	62
C	7,850	11	7·6	60
D	3,430	5	3·2	11
E	33,700	53	30	1,011
Total	50,650	76	—	3,974

Serum content of activable PF.—The total of 3974 × 10^3 EBD obtained from fractions B, C, D and E of 1700 ml. of serum (Table I) is typical of the yield of PF by fractionation, and corresponds to 2338 EBD of activable PF in 1 ml.; and of this three-quarters is the PF present in B/1R/a, which we have assumed to be the characteristic PF of human serum. Elder and Wilhelm (1958) found that up to 5000 EBD of PF/Dil were activable by dilution of 1 ml. serum. From the relatively high content of the two permeability factors in serum and the similarity

of their properties (see below), we conclude that the PF in B/1R/a and PF/Dil are identical. The other PF in fresh serum, PF/Nat, differs from that in B/1R/a in respect of heat lability, time-course and suceptibility to inhibitors; it is in any event present only to the extent of about 100 EBD per ml.

Properties of Human Serum PF

The B/1R/a from human plasma corresponds, as a permeability factor, to the fraction G2/1R of the serum of the guinea-pig, rat and rabbit. The properties of human PF described below were, in most instances, determined upon both G2 and B/1R/a fractions; all definitive tests were made on B/1R/a.

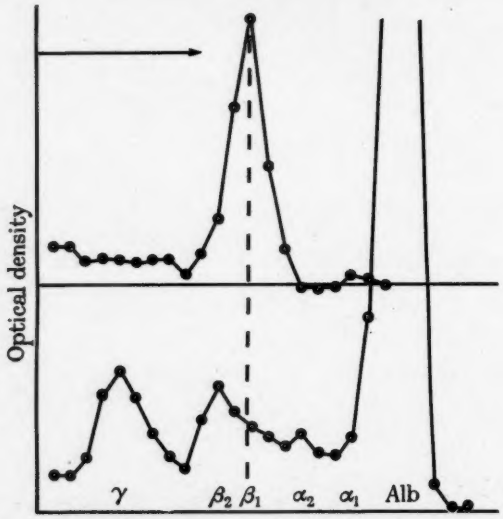

FIG. 2.—Paper electrophoretic fractionation of whole serum after the removal of fibrinogen (fraction AS: lower curve) and of purified serum PF (fraction B/1R/a: upper curve).

Protein nature.—Since during the fractionation the PF is brought down by conditions which precipitate protein but leave the small molecular components of serum in solution; since, before assay, the B/1R/a is dialysed for 3 days against buffer; and since PF activity was consistently associated with the β-globulins in all our fractionation procedures it appears that the PF is a large-molecular substance and is almost certainly protein.

Electrophoresis of B/1R/a in comparison with fraction AS shows (Fig. 2) that B/1R/a is almost entirely β_1-globulin.

Lipid content.—B/1R/a contains lipid removed by ether extraction (McFarlane, 1942); its PF potency is unaffected by this procedure. Lipid was more completely extracted by the method of Bloor, Pelkan and Allen (1922); digests for phospholipid assay were prepared by the method of Gortner (1945) and the phosphate estimated by the method of Kuttner and Lichtenstein (1930). The ratio of lipid phosphorus to nitrogen was 0·099.

Cholesterol in the lipid extract was estimated by the method of Sperry and Brand (1943), as modified by Professor N. H. Martin (unpublished). The ratio of total cholesterol to nitrogen was 2·83.

It is noteworthy that these 2 ratios are both about 35 per cent of the values for purified human β_1-lipoprotein (Oncley, Gurd and Melin, 1950), suggesting that B/1R/a is a mixture of lipo-protein and non-lipid protein.

Stability to storage, heat and pH.—After one month at — 10° a 1 per cent solution of B/1R/a lost 75 per cent of its potency. At — 25° it retained its full potency for 4 months.

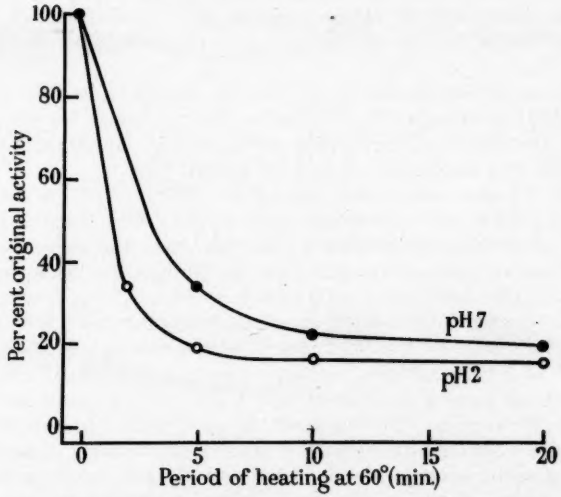

FIG. 3.—The heat-lability at 60° of human serum PF (fraction B/1R/a) at pH 2 and at pH 7.

A 0·2 per cent solution of B/1R/a in saline at pH 7·0 and at pH 2·0 rapidly lost about 80 per cent of its activity at 60°. The remaining 20 per cent was unaltered by more prolonged heating at this temperature (Fig. 3).

Samples of 0·3 per cent B/1R/a were adjusted to various pH values with 0·3 M HCl, $NaHCO_3$ or NaOH, using a glass electrode for pH measurement and diluted to a final concentration of 0·2 per cent. After 30 min. at 37° the mixtures were neutralized with the complementary quantity of acid or alkali and 2 volumes of phosphate buffer pH 8·0, I = 0·2. The PF was stable over the pH range 6·0 to 10·0, lost half of its activity at pH 5 and about 80 per cent at pH 2, 3 and 4. As in the tests of stability at 60°, 20 per cent of the PF appeared to be more stable than the remainder.

Low potency G2 fractions of human serum are more stable than B/1R/a. They are stable for many months at — 10° and for 2 months at 4°. Heating for 30 min. at 65° or 10 min. at 80° decreases their potency by 30 per cent and for 2 min. at 98° by 70 per cent. They are stable at pH 9, but at pH4 lose 30 per cent of their potency after 3 days at 4°.

The dosage-response of the guinea-pig to intracutaneous PF.—Human serum PF resembles the rat and guinea-pig PF in inducing a linear dosage-response, of lesion-diameter on log. concentration, with a slope of about 3·5. But whereas with guinea-pig PF the slope flattens at a lesion-diameter of about 8–9 mm., that of human PF flattens at 7–8 mm. The constant dose titrations of Miles and Miles (1952) indicated a degree of " affinity " between the PF and the skin tissues like that of the PF from sera of other species, and greater than that of histamine. In this connection high affinity presumably depends on the presence of a large number of sites in the skin capable of binding the permeability factor. The effect of such high affinity is a pronounced accumulation of PF at the site of the injection, with little subsequent spread as the original 9–10 mm. injection bleb spreads outwards ; and is manifest in the flattening of the dosage-response curve at the 8 mm. level.

The time-course of intracutaneous PF.—The increased capillary permeability induced by 9 EBD of human PF persisted at its maximum for about 3 min. and declined within 10–20 min. to normal low permeability. The time-course resembles that of histamine and of guinea-pig and rat serum PF.

The response of human skin to the serum PF.—Human G2/1 and G3, a preparation of guinea-pig G2/1, and histamine were applied by a scratch method to the skin of young adult human volunteers. To minimize the risk of cross-infection by the virus of homologous serum jaundice, the human fractions were made from the serum of a single donor who was known to have given non-infective transfusion blood on 9 previous occasions. Single, linear scratches a few mm. long were made on the volar surfaces of both upper forearms through drops of various test solutions. Each of 5 preparations (see below) were applied in 10 randomized sites within a rectangular area of skin about 8 × 6 cm. on each arm, and the width of the resulting weals averaged. The width of the weal proved to be a more consistent measure of the response than the diameter of the weal induced by a single pricking-in of the test materials, as recommended by Squire (1950), but even so the variation was large ; so that, to obtain significant differences in response, 10 replicate sites were tested in each subject. The mean responses, estimated from 90 weals in a total of 9 subjects, were as follows.

Material	Conc. (mg./ml.)	Weal (mm.)
Human G2/1	30	2·01
„ G2/1	15	1·93
„ G3	30	1·49
Guinea-pig G2/1	30	2·95
Histamine	5	4·56

G2/1 is significantly more potent than G3—the standard error of the difference (0·52 mm.) being 0·07 mm. The activity of G3 corresponds to the low, but definite, PF potency of crude G3 fractions. The difference between the responses to the two doses of G2/1 is not significant. As previously reported (Wilhelm *et al.*, 1955), guinea-pig G2/1 induces wealing in man, and, in accord with its greater PF activity in the guinea-pig, it is more potent in man than the human G2/1. Compared with histamine, the wealing activity of all the serum preparations is very low ; in spite of the fact that guinea-pig G2 has a PF potency nearly equal to that of histamine on intracutaneous injection in the guinea-pig. The high concentrations of serum PF needed for a positive scratch reaction is probably a reflection of the

high molecular weight of this protein and the great affinity of the PF for skin tissue, both of which properties would limit its penetration beyond the confines of the scratch, as compared with the small size of the histamine molecule, and (Miles and Miles, 1952) the low affinity of skin tissues for histamine.

The test dose of histamine induced a large characteristic flare as well as a weal (Lewis, 1927). The G2 preparations induced only a small flare, which was very little larger than that occurring around control traumatic weals induced by scratching through saline, *i.e.*, wealing without any striking flare reaction is a characteristic of the response to G2 preparations.

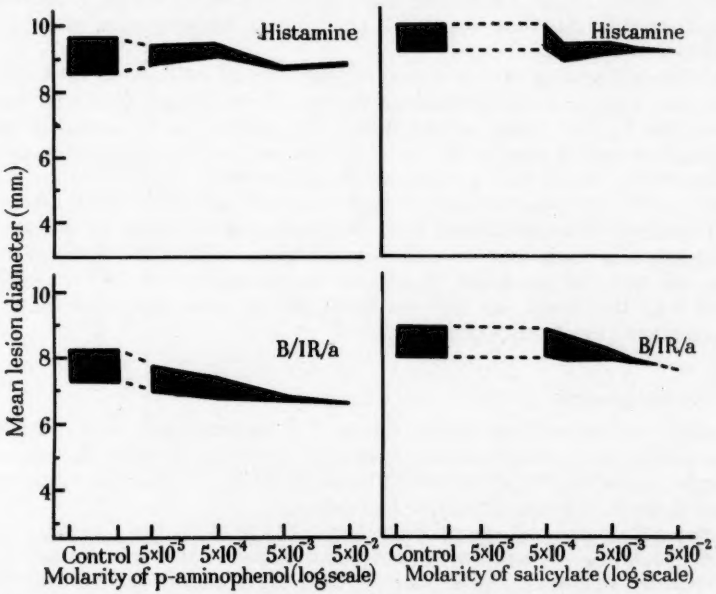

FIG. 4.—The decrease by *p*-aminophenol (left) and sodium salicylate (right) of the intensity of blueing produced (*a*) by histamine and (*b*) by human serum PF. The thickness of the lines indicate the colour of the lesions as judged by eye; the thickest line = ++++, the thinnest = very faint trace.

Susceptibility to inhibitors (guinea-pig assay).—Human PF is highly susceptible to soya bean trypsin inhibitor (SBTI—Worthington Biochemical Sales Co., New Jersey, U.S.A.), 2–3 EBD of a potent preparation of serum PF being inhibited by about 1/20th of its weight of SBTI. It is as susceptible as guinea-pig PF to guinea-pig IPF (the fraction G1S/P of guinea-pig serum). The IPF reacts relatively slowly with the PF *in vitro* at room temperature; in 4 hr. 2 EBD lose 75 per cent of their activity in the presence of 25 μg. of guinea-pig G1S/P.

Human PF is moderately susceptible to pancreatic trypsin inhibitor (PTI—Worthington Biochemical Sales Co.) 2–3 EBD being inhibited by an equal weight of PTI. It is insusceptible to ovomucoid trypsin inhibitor prepared according to Lineweaver and Murray (1947), and its activity is not diminished in guinea-pigs receiving 20 mg./kg. of mepyramine maleate intraperitoneally 2 hr. previously.

The wealing induced *in human skin* both by histamine and by the histamine-liberating compound 48/80 was inhibited when mepyramine was added to the test solutions; but mepyramine had no such effect on the wealing induced either by human or guinea-pig G2 fractions.

The action of the " antiphlogistic drugs " sodium salicylate and *p*-aminophenol was peculiar. B/1R/a containing 4–5 EBD of serum PF, and 10 EBD of histamine were each mixed with graded concentrations of the 2 drugs in saline. There was (Fig. 4) a striking decline in the intensity of the blueing in the guinea-pig with increasing concentrations of both the salicylate and the *p*-aminophenol; and over a 100- to 1000-fold range of inhibitor concentration, a slight but progressive decline in lesion-diameter such that even with the highest concentration of inhibitors it never exceeded 1·0 mm.

A decline in diameter of this degree corresponds to a 30–50 per cent loss of PF potency, but this is a small decrease compared with that produced by frank inhibitors like SBTI. Moreover the decline in potency as measured in terms of lesion-diameter occurs over a 100- to 1000-fold range of inhibitor concentrations, whereas with SBTI and PTI a comparable decline occurs with less than a 10-fold increase in inhibitor concentration; suggesting that salicylate and *p*-aminophenol are not interfering in a specific way with the reaction of the serum PF and the tissue. This suggestion is consistent with the equal susceptibility of equipotent amounts of histamine and the serum PF to similar concentrations of the two drugs. We conclude that the drugs act by diminishing the general susceptibility of the blood vessels to permeability factors.

Relation to the plasmin system

Plasmin was present in all our serum PF preparations. The distinction of PF from components of the plasmin system is more readily made in human serum than in the animal sera that we have already studied, because the system is most precisely defined in man.

Human PF proved to have a different stability from that of plasmin, plasminogen, and plasminogen proactivator and our best preparations contained no spontane activator. Moreover, unlike plasmin, PF was not absorbed by fibrin. Preparations of B/1R/a were adjusted to pH 2 with 0·3 M HCl, incubated for 10 min. at 37° and neutralized by 0·3 M $NaHCO_3$. The percentage decline in potency was

Plasmin . .	18
Plasminogen .	12
PF . . .	75

The PF was also more heat labile than either plasmin or plasminogen. The relative percentage losses of activities from a 0·2 per cent solution of B/1R/a heated at pH 7 at 60° for 10 min. were

Plasmin . .	41
Plasminogen .	57
PF . . .	82

The fairly high apparent lability of plasminogen is possibly due to a loss of pro-activator.

The different effects of incubation at pH 5·5 on the PF and plasminogen proactivator are illustrated in Fig. 5.

A differential absorption by fibrin of plasmin, plasminogen and PF from solutions of B/1R/a, was carried out as follows. A 0·05 per cent solution of B/1R/a was adjusted to pH 7·2 with a trace of buffer, a small sample was removed and 10 per cent (v/v) of a 2 per cent solution of highly purified human fibrinogen was added. Slow clotting occurred at room temperature. The clot was compressed with a glass rod and the fibrin removed. A sample of the solution was set aside and the entire process repeated. In all 5 additions of fibrinogen were made. The relative

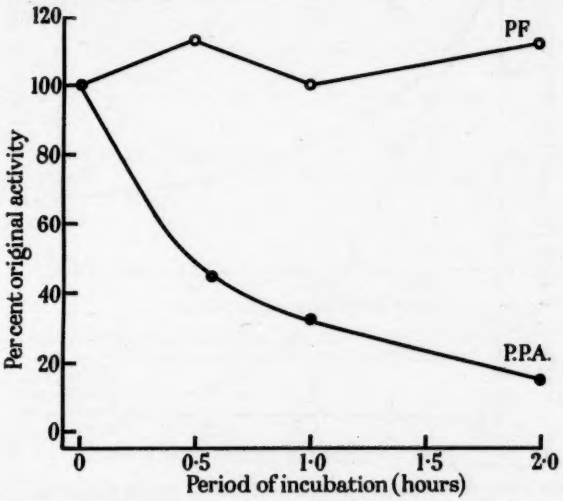

FIG. 5.—The relative stability at pH 5·5 and 37° of the serum PF and the plasminogen pro-activator (P.P.A.) in fraction B/1R/a.

PF, plasmin and plasminogen contents of the 6 samples (Fig. 6) clearly indicate that PF cannot be identified exclusively with these components of the plasmin system.

Plasmin as a permeability factor

The residual 20 per cent of PF activity found in preparations of B/1R/a subjected to heat or low pH suggested the presence of a second permeability factor in these preparations. Stability at pH 2 is characteristic of plasmin and, as Fig. 7 shows, the caseinolytic activity of B/1R/a is only slightly affected by treatment at pH 2, whereas the PF activity rapidly falls to the 20 per cent level and is then fairly stable. The acid stable PF is as susceptible to SBTI as the normal PF. This minor PF component, which is relatively stable and susceptible to a protease inhibitor, may therefore be plasmin itself. Even if this is so the PF effect of the plasmin is not simply a correlative of its general proteolytic ability. The PF and protease potencies of B/1R/a, of B/1R/a held at pH 2 and 37° for 30 min., of B/1R/a treated with fibrin as above and of crystalline trypsin (Armour Labora-

tories, London) are compared in Table II. The relative potency of B/1R/a is in each case taken as unity. The ratio of PF to protease activity of 0·2, is characteristic of the acid-treated plasmin preparations but the ratio for trypsin is only 0·001. If, then, the more stable PF component of B/1R/a is plasmin, then the substrate of the proteolytic permeability factors is a highly specialized one; and the components of the peptide bonds which are split to bring about the increase in permeability must be different from components of the bonds which are split during the proteolysis of casein.

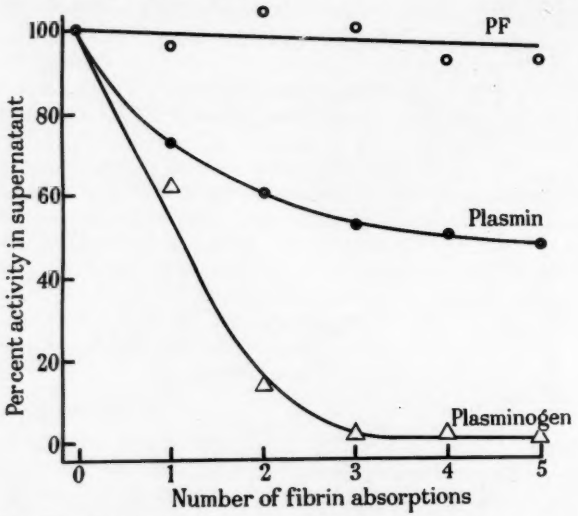

FIG. 6.—The effect of successive absorptions by fibrin on the PF, plasmin and plasminogen content of fraction B/1R/a.

TABLE II.—*The Relative PF and Proteolytic Activities of Preparations of B/1R/a and Trypsin*

Substance	Treatment	Presumed constitution of preparation	Relative activity		Ratio of activities
			PF	Protease	PF : Protease
B/1R/a	*Nil*	PF + plasmin	1·0	1·0	1·00
„	Acid	Plasmin	0·2	0·8	0·25
„	Fibrin	PF + some plasmin	0·9	0·5	1·80
Trypsin	*Nil*	—	2·2	1600	0·001

The Properties of IPF, the Inhibitor of Human PF

Human IPF was precipitated in fraction C and D of the scheme set out in Fig. 1. Fraction E was inactive; fraction C and fraction D, held for 15 min. at room temperature with 18 μg./ml. of B/1R/a, both inhibited PF (Fig. 8), but fraction D was the more potent. The rise in lesion-diameter on the right of the figure suggests that both C and D contain small amounts of a PF—about 7 EBD/mg. of protein in D. The properties of the IPF were investigated using fraction D at

concentrations of 250 μg./ml. or less to avoid interference by this PF. *In vitro*, human IPF, like that of guinea-pig and rat, at room temperature acts relatively slowly and progressively on its homologous PF. For example, 15 μg. of fraction

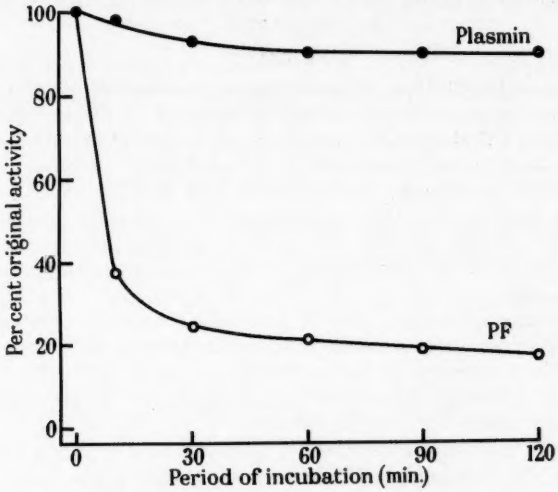

FIG. 7.—The relative stability at pH 2·0 and 37° of the PF and plasmin content of fraction B/1R/a.

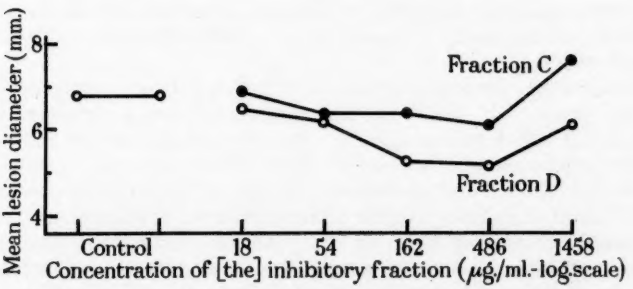

FIG. 8.—The inhibition of the human serum PF in fraction B/1R/a by the IPF in fractions C and D.

D decrease the activity of 2 EBD of PF by 50 per cent in 2 hr. Although guinea-pig IPF was active against human PF, the human IPF in fraction D had no demonstrable action on guinea-pig PF. The percentage inhibition of approximately 2 EBD of PF, after 2 hr. at room temperature with 25 μg. of the two IPF preparations, was as follows.

	Inhibitor	
	Human D	Guinea-pig G1S/P
Human B/1R/a . .	75	60
Guinea-pig G2/1R . .	0	60

Fraction D is a very impure preparation of human IPF and a more extensive exploration of the properties of this inhibitor must await its further purification. It is of interest to note however that in fraction D we also found a slowly and progressively-acting inhibitor of trypsin and a feeble inhibitor of plasmin.

SUMMARY

Human plasma, like the sera of guinea-pig, rat and rabbit, yields on fractionation a potent capillary permeability factor (PF). One ml. of plasma yields about 2300 guinea-pig EBD of PF, the greater part of which in respect of indiffusibility through cellophan, time-course, and susceptibility to inhibitors, corresponds to the PF/Dil activated by dilution of human serum (Elder and Wilhelm, 1958).

A scheme is described for the separation of this factor by solvent fractionation, as a preparation consisting of a lipid rich β_1-globulin which is virtually homogeneous on electrophoresis. Extraction of the lipid does not affect the PF activity of this preparation.

The PF is more labile than that in guinea-pig, rat and rabbit sera. Some 80 per cent of the PF activity is rapidly lost on heating to 60° or on acidifying to a pH below 5. The remaining 20 per cent of the activity is much more stable even at 60° and pH 2. Although the labile PF is not any of the components of the plasmin system, the more stable PF may be plasmin.

The pattern of inhibition of human PF is similar to that of the PF in guinea-pig, rat and rabbit. It is strongly inhibited by soya bean trypsin inhibitor and to a lesser extent by pancreatic trypsin inhibitor, suggesting that its action involves proteolysis. It is not affected by the more specific trypsin inhibitor ovomucoid, nor by the anti-histamine drug mepyramine. The anti-phlogistic drugs sodium salicylate and *p*-aminophenol also inhibit the permeability effect of human PF but apparently as the result of a general non-specific decrease in the reactivity of small blood vessels.

The fractionation process also separates a preparation containing an inhibitor (IPF) of the human PF, which *in vitro* acts slowly and progressively on human PF but does not affect guinea-pig PF. By contrast guinea-pig IPF acts equally well on both guinea-pig and human PF. The IPF preparations from human serum also inhibit both the PF and proteolytic activity of trypsin and plasmin.

In 1929 Krogh, in order to explain certain features of inflammation in man which were not readily explicable in terms of histamine, postulated the existence of an " H-colloid " of large molecular weight and low diffusibility, which mediated vasodilation and increased permeability of the vessels, without inducing the axon-reflex flare characteristic of the response to histamine. The material separated by us has the two physical characteristics required of this " H-colloid ", and, as Stewart and Bliss (1957) also demonstrated with dilutions of fresh human serum, it increases the permeability of the capillaries of human skin producing a weal, with very little flare reaction.

We are indebted, through Dr. A. C. Cunliffe, to students of King's College Hospital Medical School who gave blood for this investigation, and to our colleagues Drs. A. E. Mourant and D. Parkin for harvesting the blood ; to our colleagues Drs. Margaret Mackay and R. A. Kekwick for advice on the fractionation techniques and for the electrophoretic assay of serum fractions in the Tiselius apparatus ; and to the Nuffield Trust for financial help in this investigation.

Much of this work was reported by one of us (P. J. M.) in a thesis accepted by the University of London for the degree Ph.D.

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OBSERVATIONS ON MAST CELL CHANGES, HISTAMINE RELEASE AND LOCAL TISSUE DAMAGE IN RATS FOLLOWING X-IRRADIATION

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IN a previous paper (van den Brenk, 1957) evidence was presented which failed to substantiate the view that acute lethality in rats caused by total body irradiation was mediated by the release of histamine induced by irradiation. It was shown that radiation lethality in rats, depleted of histamine with Compound 48/80, was significantly increased. This substance, besides reducing the histamine content in most rat tissues to very low levels (Feldberg and Talesnik, 1953), causes rupture of mast cells (Riley and West, 1955) in common with other synthetic histamine depleting agents and antigenic substances. It has been reported that skin becomes refractory to local injections of 48/80, but not to histamine, 20 hr. after irradiation with β-rays (Ungar and Damgaard, 1954). The present studies were undertaken to determine whether skin damage due to irradiation is modified by histamine depletion of the animal; whether irradiated skin is refractory to inflammatory stimuli; whether total body irradiation modifies the shock-like response to a large dose of Compound 48/80 and whether mast cells, irradiated to high dosage with X-rays, still respond in a typical manner to 48/80 *in vivo*.

METHODS

In order to study the changes in the morphology of mast cells after X-irradiation of the skin or administration of Compound 48/80, 32 adult male laboratory stock Canberra Black rats (weighing 120–160 gm.) were equally divided into 8 groups. In 7 groups, 3 rats received local irradiation to the dorsal skin using a Berylium Window X-ray tube operated at 30 kV, 10 cm. F.S.D 5 MA, and dosage rate 956 r/min. measured in air; one rat served as control. The maximum area of skin was irradiated which could be uniformly centred under a 7 cm. diameter lead cut out. A single skin dose of 4000 r, calculated with back scatter, was given under pentobarbitone sodium anaesthesia. The quality of the beam being such as to yield a half value dose of 2000 r at 2 mm. depth in the tissues.

The 7 groups of rats were killed by cervical fracture; the irradiated rats 1 hr., 24 hr., 5 days, 14 days, 4 weeks, 8 weeks and 12 weeks respectively after irradiation. Subcutaneous tissue spreads were immediately prepared from each animal, fixed in 80 per cent alcohol and stained for mast cells with toluidine blue, methylene blue or thionine according to the technique of Riley and West (1955). Each tissue spread was scrutinised for mast cells and the morphological appearances studied. The extreme variations in mast cell content for different spreads noted by Kelenyi (1953) and Smith and Lewis (1953) made a total and differential mast cell count an unreliable procedure. Therefore, mast cells from selected spreads for certain groups were counted and differentially scored according to the morphological variations observed to confirm the qualitative findings.

The remaining 4 rats were given intraperitoneal injections of Compound 48/80. Two rats received each 100 μg. 48/80 diluted in normal saline, followed after 12 and 24 hr., by

further injections of 200 μg. 48/80. One hour after the last injection the rats were killed and subcutaneous spreads were prepared and stained for mast cells. In addition spreads of mesentery and omentum were made. These were defatted in ether-alcohol and similarly stained. The remaining 2 rats received a 5 day course of chronic depletion with Compound 48/80, in accordance with the specifications of Feldberg and Talesnik (1953), with increasing daily increments of 100 μg. 48/80. The animals were then killed; similar spreads were made and stained.

In order to study the effect of a large dose of Compound 48/80 after total body irradiation, 5 adult male Canberra Black rats (average weight 200 gm.) were subjected to a high single dose of 2000 r total body irradiation (mean dose calculated at a depth of 2 cm.) from an X-ray tube operated at 240 kV., 15 MA, 30 cm. FSD, and HVL 1·0 mm.Cm. This dose represents an LD100 in 5 days. A similar group of 4 rats (average weight 219 gm.) served as control.

Each irradiated and control rat 24 hr. after irradiation received 800 μg. 48/80 and their reactions were recorded. The injection was repeated in surviving rats after a further 24 hr. and reactions again observed.

In order to study local reactions of irradiated skin to various agents the dorsum and one flank of 12 adult albino male rats (200 gm.) were closely clipped with an electric hair clipper 24 hr. preceding irradiation, care being taken to avoid tiny lacerations and abrasions. The skin of one flank was irradiated with X-rays from a Beryllium window tube using the same factors as for the experiment on mast cell changes. A single dose of 4000 r (half value depth dose at 2 mm. in tissue) was given to a 3·5 cm. circular field through a lead cut-out. The irradiated area was carefully encircled with India ink. The opposite flank served as control. The animals were anaesthetized during irradiation with intraperitoneal pentobarbitone sodium.

Twenty-four hours after irradiation 9 rats received intradermal injections into the centre of the irradiated area and at the same site on the opposite unirradiated flank. Three rats received 0·1 mg. histamine acid phosphate; 3 received 1 mg. Compound 48/80 and 3 received 50 per cent croton oil in mazola oil. The volume injected each time was 0·1 ml. The remaining 3 rats were given a second irradiation to a 1 cm. circle of skin in the centre of the previously irradiated zone and a similar exposure was given to the opposite unirradiated flank. The source and physical factors for this second radiation exposure were similar to those used in the primary one, but the skin dose given was 10,000 r to the 1·0 cm. diameter field. Subsequently the skin changes were observed and charted for selected times after each treatment.

In order to study the morphological changes of 48/80 on irradiated mast cells 12 Canberra Black rats (120–155 gm.) were anaesthetised with intraperitoneal pentobarbitone sodium. Three rats served as controls and were not irradiated. In the other 9 rats the peritoneal cavity and its content were irradiated from the diaphragm to the pelvic outlet to a single mean dose level in tissues which in six rats was 2000 r and in 3 rats 4200 r falling to 4000 r. Irradiation was given whilst the rats were lying on their backs on a wax block, through an oblong lead cut-out, using compression with the closed face of the cone to aid homogeneity of dosage. A 240 kV X-ray source was used.

Twenty-four hours after irradiation or mock anaesthesia the animals received the following treatment: The 3 control rats and 3 rats irradiated with 2000 r received 24 hr., 48 hr. and 56 hr. after the anaesthesia or irradiation intraperitoneal injections of 100, 200 and 500 μg. of 48/80 in normal saline solution (500 μg. 48/80 per ml.). The 3 other rats irradiated with 2000 r received at the same times intraperitoneal injections of the respective volume of saline solution. One hour after the last injection the rats were killed. Tissue spreads of the abdominal subcutaneous tissue of the mesentery and of the omentum were prepared, fixed and stained for mast cells with polychrome methylene blue, toluidine blue or thionine. The 3 rats irradiated with the higher dose received a single intraperitoneal injection of 800 μg. of 48/80 24 hr. after irradiation. At this time the abdomen of the rats was swollen and the injection caused prostration and shock-like symptoms. One rat died within 1 hr. of the injection. Spreads of the abdominal subcutaneous tissue, the mesentery and omentum were prepared at once and stained for mast cells. Similarly prepared spreads were made in the other 2 animals 3 hr. after injection, when they had recovered and were killed.

In order to study irradiation reactions in histamine depleted skin, 20 male albino rats were divided into 2 groups of litter mates (mean weights, 122 (±19) gm. and 116 (±13) gm. respectively). One group received daily intraperitoneal injections of 0·5 ml. normal saline;

the other received twice daily for 20 days intraperitoneal injections of Compound 48 to 80 commencing with 100 μg. per injection and increasing the dose by daily increments of 100 to 500 μg. which was then continued to the end of the experiment. On the third day the dorsal skin of the animal was carefully and closely clipped with electric clippers. On the fourth day 3 hr. after the first injection, the rats were anaesthetised with intraperitoneal pentobarbitone sodium and a circular field of skin, 3·5 cm. in diameter, situated centrally in the distal portion of the dorsal trunk, was irradiated through a cut out in a lead shield. The opening in the cut-out was covered with tightly stretched thin cellulose acetate film to give uniform flattening of the skin to be irradiated.

A Beryllium window X-ray tube was used, operated at 30 kV., 5 MA, 10 cm. FSD, giving an output in air of 240 r/sec. and an H.V.D. in tissue of 1 mm. All animals received a single skin dose of 3000 r.

At selected times after irradiation the skin reactions were observed and recorded independently by two observers. Observations were performed in daylight without the aid of filters. Where observers differed on a given reaction, the lowest value was scored.

Three weeks after irradiation the changes in hair growth in the irradiated zones (epilation) were charted. However, clipping of hair does not standardise growth activity in hair follicles in the same way as plucking (Chase, 1951). The latter was not allowable, for obvious reasons, in assessing skin reactions. The lower dosage (3000 r) was chosen as a result of past experience. Less intense reactions permit more confident scoring of the intensity of a skin change. Handling of animals, necessary for the examination of abdominal skin, makes the abdominal skin less satisfactory than dorsal skin despite its thinness and greater sensitivity to irradiation.

RESULTS

Effect of Compound 48/80 *on mast cells*

The results obtained agree with those recorded by Riley and West (1955). Riley (1953) distinguished between two types of mast cells, Type I and Type II (Fig. 1 and 2); both types were absent in tissue spread in animals which had received large doses of 48/80 (Fig. 4). High power views showed a wide spreading distribution of typical fine metachromatic granules in the tissues (Fig. 5), often with a "concentric" disposition, related to the cell of origin. There was increased basophilia of the tissues and many granules were engulfed by fibroblasts as described by Higginbotham *et al.*, (1956). The granules retained the even, fine texture seen in normal mast cells and stained a bluish purple with polychrome methylene blue stain. With lower doses vacuolation of mast cells was seen. Partial loss of granular material gives a patchy ghost-like appearance to such incompletely depleted cells, which may be mistaken for changes in staining of certain of the remaining granules.

Effect of irradiation on mast cell morphology

The changes which take place in mast cell morphology at various times after irradiation must be distinguished from changes brought about by the technique of preparing the spreads. Many so-called "ruptured" mast cells result from mechanical trauma as shown by Kelenyi (1953) and may be mistaken for radiation induced changes. "Ruptured" cells of this type, which have been illustrated in various papers and attributed to irradiation damage and other causes, were found in both control and irradiated preparations. They were ignored as a radiation effect.

Departures from the normal Riley types I and II mast cell morphology, seen in irradiated tissues, were occasionally seen in unirradiated spreads. Irradiation changes were observed as early as 1 hr., they were maximal at 24 hr. and remained

so for the next 4 days. In this condition, clear vacuolation, the typical effect of Compound 48/80, occurred rarely. A common finding was swelling of mast cells, loss of normal oval outlines and substitution of irregular shaped cells with deep indentations. The granules and nucleus appeared normal. Frequently a discrete portion of the cell cytoplasm was separated off from the main cell body. Another frequent change was irregularity in the texture and conglomeration of granules to form giant metachromic inclusions (Fig. 4 and 5). Metachromic inclusions paralleled those seen *in vitro*, by Paff and Bloom (1949) termed clasmatosis and considered to have developed by solution of granular substance into a clear vacuole. Nuclear swelling and apparent absence of nuclei was commonly seen in such clasmacytes.

The swelling of cytoplasm and nucleus of such cells progressed and resulted in the appearance of rupture as shown in Fig. 3. There was a difference between this change and that seen in cells subjected to 48/80. Comparison of Fig. 3, 4 and 7 with Fig. 5 and 6 shows this difference. After irradiation the granules and inclusions of " bursting " clasmacytes do not widely disperse through tissues as after 48/80 and the cell remains clearly outlined in lower power views. This appearance when seen in normal tissues is attributed to natural senescence and death of mast cells.

A less common change after irradiation was pyknosis of cells. The cell outline consisted of a shrunken irregular mass of deeply staining metachromic material, sometimes vacuolated and often showing no granules in high power views.

With high doses of irradiation (4000 r) up to 50 per cent of the cells are irregular. They have swollen outlines in which the metachromic material has become confluent, giving a light purple colour with polychrome methylene blue stains which sharply contrasts with the deeper blue of cells appearing normal (Fig. 7). The cytoplasm also contains deeper staining confluent patches, rather sharply defined, but devoid of normal granular texture.

A striking feature of the effects of irradiation on mast cells is the persistence of cells, normal in appearance, amidst cells of altered morphology (Fig. 3 and 7). Such " normal " cells sometimes show early cytoplasmic clefts (Fig. 3), which appear to precede the more advanced changes.

The changes at various times after irradiation closely follow those described by Kelenyi (1953). One hour after irradiation there was a large percentage of abnormal cells. At this time the abnormal changes largely consisted of swelling, irregularity in outline and the appearance of clefts. Spreads obtained 24 hr. and 5 days after irradiation showed a higher proportion of cells with alterations in granules, *viz.*: giant granules and metachromic homogeneous cytoplasmic staining. Fourteen days and as late as 4–8 weeks after irradiation abnormal mast cells were still in excess, but there was also a general reduction in the total number of mast cells. By 8 weeks, numerous small mast cells had appeared, particularly in relation to vessel walls (Type I cells). The irregular distribution of such areas with small mast cells was striking and conformed with the description given by Riley and West (1955) as evidence for mast cell regeneration from mesenchymal precursors. However little evidence of " refilling " of mature cells (Type II) with granules was observed, as described for recovery after depleting agents. The spreads throughout the post-irradiation periods always showed areas of mast cells of normal appearance of both Types I and II and the abnormal changes

were often confined to restricted areas in the tissue. At 12 weeks after irradiation, some subcutaneous spreads showed a remarkable increase in mast cell density, the cells were mature in appearance and often arranged in tandem—a change suggestive of repeated division (Riley and West, 1954).

Effect of 48/80 *in rats subjected to total body irradiation*

An intraperitoneal injection of 800 μg. of Compound 48/80 produced the same shock-like reactions in 4 control rats and in 5 rats which 24 hr. earlier had received 2000 r total body irradiation. Three minutes after the injection 2 rats of each group were prostrate and cyanosed and 2 min. later all 9 rats were prostrate and lying in isolation. Death occurred in one irradiated rat 90 min. after the injections, whereas 3 control rats died, one at 30 min. and 2 at 60 min. after the injection. The remaining rats recovered within 3 hr. A second intraperitoneal injection of 800 μg. 48/80 was given 24 hr. after the first injection to the remaining 5 rats. The result was rapid prostration in all animals, but none died and recovery began after 45 min. and was apparently complete in 90 min.

Local response of irradiated rat skin to histamine, Compound 48/80, *croton oil and renewed irradiation*

In all animals the control and irradiated skin showed the typical reaction to an intradermal injection of histamine. A few minutes after the injection a central wheal appeared with pseudopodia, slightly larger in the irradiated skin. The wheal was surrounded by an area of erythema. In the unirradiated skin this erythematous zone was small and localised, in the irradiated skin it was more intense and much larger. In 2 of 3 animals the erythema spread to the whole of the 3·5 cm. diameter irradiated circle of skin, which became clearly delineated by the erythema. Previously it had been impossible to identify the irradiated field at this time (24 hr. after irradiation). Fading of the reaction seemed equal on both flanks after approximately 5 min.).

The effect of intradermal 48/80 was similar to that of histamine. The wheal and the erythematous surround again appeared on both flanks, but on the irradiated skin the erythema was more widespread and again in 2 of 3 animals it outlined the irradiation field. Twenty-four hours after the injection a small wheal was still present on both flanks ; it was surrounded by a small erythematous halo on the unirradiated side and by a more diffuse, but faint, erythema on the irradiated flank.

A minute after the intradermal injection of croton oil an erythematous flare developed around the site of injection and in the irradiated skin the whole irradiated zone was outlined. Subsequently the erythema quickly disappeared, no definite wheal being formed, but 3 hr. later there was a central nidus of necrosis at the injection site surrounded by swelling—more pronounced in the irradiated skin. No erythema was present at this stage. Twenty-four hours after the injection the necrotic nidus was still present, surrounded by swelling in both flanks, together with some erythema of the skin. The swelling was greater in irradiated skin and persisted. Eight days after the injection the swelling had increased on both sides, but was more pronounced in the irradiated skin. By the tenth day a severe irradiation reaction was present and breakdown of injection site was taking place in both irradiated and non-irradiated skin.

The irradiated skin responded to a second irradiation exposure in an essentially normal manner. The effect was consistent with the conclusion that an additive effect occurred. The central zone could be identified readily on both irradiated and control flanks and over the next 10 days increased in severity, resulting in breakdown of epidermis (50–100 per cent. in the central zone on both flanks). The reaction on the primarily irradiated flank was more severe.

Response of irradiated mast cells to Compound 48/80

The results are set out in Table I and illustrated from typical tissue spreads in Fig. 8.

TABLE I.—*The Effect of Subacute Depletion with Compound* 48/80 (3 *Doses of* 100 *μg., 200 μg., and* 500 *μg.* 48/80 *in* 48 *hr.), and Acute Depletion (Single Dose* 800 *μg.* 48/80) *on Mast Cells of Irradiated and Unirradiated Tissues*

	Group	Treatment	Effect on mast cells
I	Non-irradiated control group	Subacute depletion	Typical widespread liberation of granules in the tissues.
II	Irradiated (mean dose 2000 r) group	Saline injections	Typical mast cell damage without generalised liberation of granules in tissues.
III	Irradiated (mean dose 2000 r) group	Subacute depletion	Widespread liberation of granules, but certain mast cells showing typical signs of irradiation damage, remain " unruptured ".
IV	Irradiated (mean dose 4000 r) group	Acute depletion	Increased proportion of " unruptured " but abnormal cells showing typical radiation changes. Widespread dissemination of granules in tissues.

In irradiated tissues treated with saline, typical radiation changes already described are observed in mast cells. Such tissues also show the typical mast cell " rupture " in response to Compound 48/80, but disorganised cells remain " unruptured " in spreads of all tissues examined. The liberated granules of irradiated cells were considerably larger than those of normal cells similarly subjected to 48/80. The clasmocytes with giant inclusions and greatly swollen nuclei also responded to 48/80. The cells which remained unaffected by 48/80 were the pyknotic types and the irregular cells containing homogeneous metachromic cytoplasm with irregular large but non-granular inclusions. Even a huge dose of irradiation (4000 r) which, when given locally to rat tissues causes a high proportion of complete skin breakdown, does not interfere with the apparent physiological behaviour of most tissue mast cells as indicated by reaction to a histamine depleting agent.

Irradiation reactions in skin of histamine depleted rats

The results obtained are recorded as a histogram in Fig. 9 and the areas under the histogram have been integrated and graphically recorded in Fig. 10. In Table III the effect on hair growth (epilation) observed in non-depleted and depleted animals is recorded. It will be seen that depletion of the skin histamine in

rats with 48/80 does not lessen the intensity of the radiation reaction in skin. In fact, the secondary erythema appeared earlier in histamine depleted animals. Recovery of hair growth 3 weeks after irradiation was more complete in controls than histamine depleted animals.

TABLE II.—*Recovery of Dorsal Hair Growth in Rats Irradiated by a Single Dose of 3000 r, X-rays to a 3·5 cm. Diameter Field. Group I Previously Treated with Saline Injections; Group II, Previously Treated with Compound 48/80*

	Group I. Number of animals	Group II. Number of animals
Active hair growth	2	1
Retarded hair growth (partial epilation)	8	5
No apparent hair growth (complete epilation)	0	4

DISCUSSION

The results give no support for the hypothesis that chronic depletion of skin histamine in the rat with Compound 48/80 results in a lessening of radiation erythema or radiation change. Whilst the results do not afford statistical proof that histamine depletion intensifies radiation damage to skin, it seems significant that the observations of skin change recorded consistently showed a more intense reaction in the histamine depleted animals and that epilation was also marked in the latter. The results thus fail to provide support for the view that the release

EXPLANATION OF PLATES

FIG. 1 and 2.—Normal mast cells in subcutaneous tissue spreads in the rat. Riley Type I cells (Fig. 1), Riley Type II cells (Fig. 2). Methylene blue stain. × 615.

FIG. 3.—Abnormal mast cells in subcutaneous tissue spread in rat following irradiation (24 h. after 2000 r X-rays)—early swelling, fragmentation and formation of clefts (A). Localised dispersion of granular contents and giant inclusions which accompanies cell degeneration (B), differs from wide spread dispersion of mast cell granules caused by a histamine depletion agent (compare with Fig. 5 and 6). Methylene blue stain. × 115.

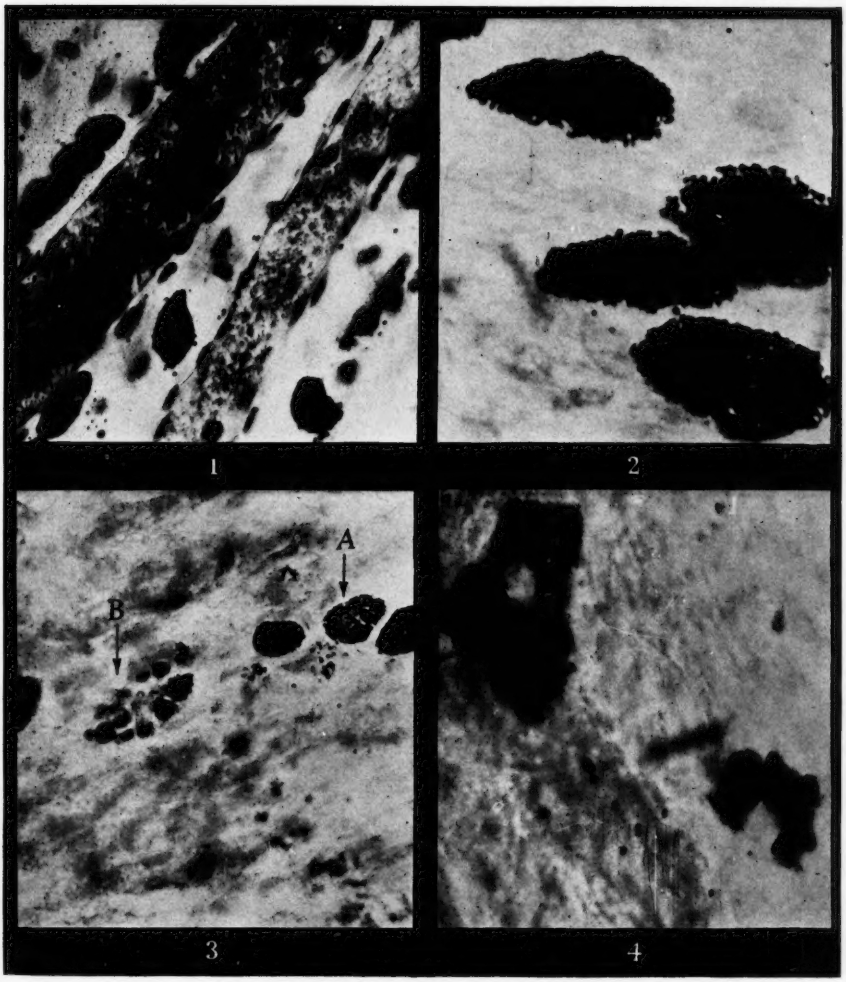
FIG. 4.—Two irradiated mast cells in subcutaneous tissue spread, the one showing cytoplasmic swelling, irregularity of outline and coalescence of granules into irregular masses, the other showing giant metachromic inclusions in cytoplasm, with eccentric nucleus. Methylene blue stain. × 615.

FIG. 5.—Subcutaneous tissue spread of unirradiated rat following subacute depletion with Compound 48/80. No intact mast cells seen in low power view. Mesenchymal tissues stain deeply due to increased basophilia. Methylene blue stain. × 115.

FIG. 6.—High power view of Fig. 5. All mast cell granules widely dispersed in tissue, but their concentric distribution outlines the original intact cells of origin. Methylene blue stain. × 615.

FIG. 7.—Spread from abdominal subcutaneous tissues of rat, 48 h. after 2000 r X-rays. Mast cells normal in appearance, (A) interspersed with cells, (B) showing typical signs of irradiation damage, namely, swelling, irregularity and abnormal staining. Methylene blue stain. × 115.

FIG. 8.—Subcutaneous tissue spread from rat, irradiated with 2000 r X-rays, and subjected 48 h. later to subacute depletion with Compound 48/80. Typical widespread dispersion of granules in tissues, following rupture of most mast cells. However, two irregularly staining cells, adjacent to blood vessel, remain unruptured. Dispersed granules are larger and basophilia of mesenchymal tissues is less pronounced than in unirradiated tissues subjected to 48/80 (compare with Fig. 5). Methylene blue stain. × 115.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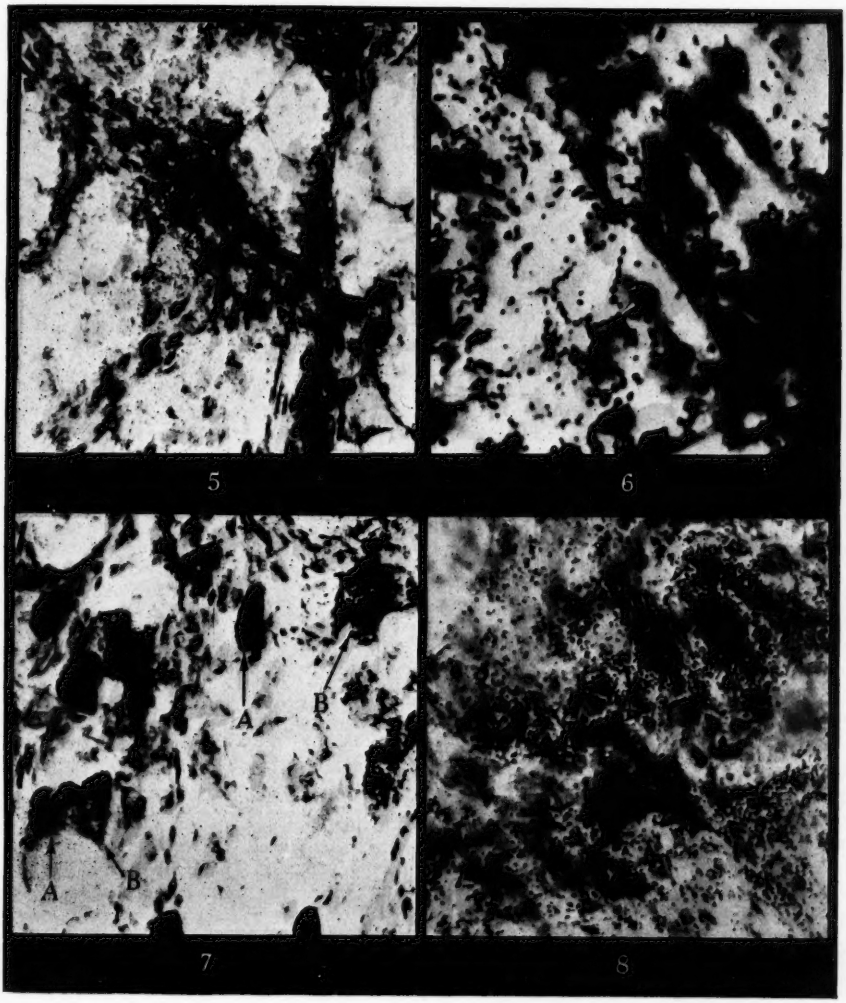

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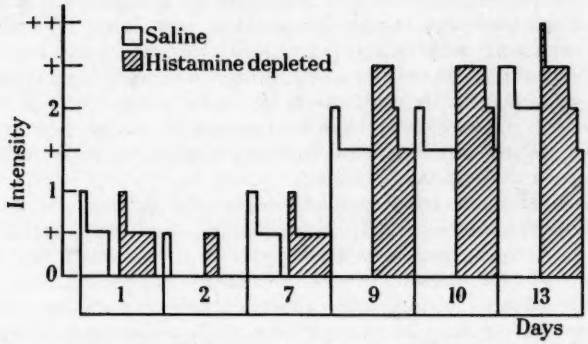

FIG. 9.—Histogram showing the intensity of the radiation reaction in skin of control and histamine depleted rats, following a single skin dose of 3000 r (30 kV. X-rays, 5 MA, 10 cm. FSD, HVD in tissue 1 mm., 240 r/sec in air output). The reactions have been scored in accordance with the following grading:

0	No visible reaction.
0+	Perceptible erythema.
1	Definite erythema.
1+	Severe erythema.
2	Dry desquamation.
2+	Partial breakdown of skin (30 per cent surface area).
2++	Partial breakdown of skin (30–50 per cent surface area).

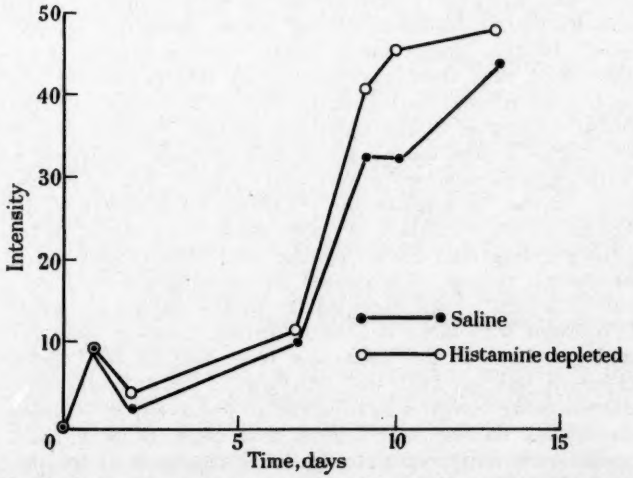

FIG. 10.—Graphical representation of histogram in Fig. 9. The areas under the histogram have been integrated, and the intensity of the reaction plotted in arbitrary units, for control and histamine depleted rats.

of histamine, which results from irradiation, plays a significant role in causing roentgen erythema and local tissue damage, as postulated by Ellinger (1951). The findings of Ungar and Damgaard (1954) that rat tissues, irradiated with β-rays from ^{90}Sr, are refractory to a histamine depleting agent (48/80) over a period, 20 hr. to 10 days after irradiation, is not supported by our findings or by those of Eisen and Wilson (1957) which appeared since the work reported here was completed. These authors used the local blueing reaction for rats injected intravenously with pontamine sky blue.

Whilst tissue histamine appears to be temporarily reduced after local and total body irradiation (Eisen *et al.*, 1956) and whilst the total mast cell content of rat subcutaneous tissues also decreases (Kelenyi, 1953 ; Conte *et al.*, 1956), it appears that the mast cell is a very radio-resistant entity. Tissue doses of 2000–4000 r X-rays, which locally administered even jeopardise viability of skin, fail to cause significant morphological changes in a high proportion of mast cells and such cells can behave in a normal way to a depleting agent. This has been demonstrated by observing the systemic effects of 48/80 after total body irradiation and by examining the morphological changes after irradiation in mast cells in subcutaneous tissues, mesentery and omentum. The reduction in systemic symptoms seen with 48/80 in animals, previously subjected to total body irradiation, is explained by a reduction in functioning mast cells. Morphological studies have shown that this reduction is essentially due to those mast cells showing gross signs of radiation damage. Simple coalescence of granules to form large inclusions (3–10 μ in size) and clasmatosis, cell irregularity, cytoplasmic vacuolation and nuclear swelling, all fail to prevent the typical morphological response of the affected cell to 48/80 and suggest that such grossly altered cells are still viable.

The morphological dissimilarity between the mast cell response to irradiation and to a depleting agent is supported by other findings. Compound 48/80, whilst causing histamine depletion of rat tissues, does not release heparin in rats (Mota *et al.*, 1953) or dogs (Quivy, 1955). This is surprising in view of the of the demonstration that mast cell granules are rich in heparin (Jorpes, 1946). Also, heparin has an affinity for histamine *in vitro* sufficient to explain the high concentration of histamine in the mast cell granule (Sanyal and West, 1956). On the other hand, irradiation causes a haemorrhagic diathesis and changes consistent with heparin release (Smith and Lewis, 1953). Paton (1956) has drawn attention to the " non-releasable " histamine fraction in skin (10 per cent in rats), apparently not contained by mast cells, but present in mast cell free epidermis. He suggests that ribose nucleic acid (RNA) may form a complex with histamine base in tissues. Under such circumstances cell damage conceivably liberates local histamine by displacement in such a complex ; whilst histamine liberators do combine with RNA *in vitro* to form a complex, they are apparently unable *in vivo* to displace the histamine contained in the " non-releasable " fraction. It seems possible that the reduction in tissue histamine reported to follow irradiation may involve a substantial reduction of " non-releasable " histamine due to cell damage. It follows that estimations of tissue histamine following irradiation in animals, naturally low in mast cell content and releasable histamine, *viz.*: the guinea-pig, dog and man, may provide more significant data than data obtained from rats.

That a reduction in tissue histamine and degranulation of mast cells by a depleting agent results in a decreased capacity on the part of the organism to

react to irradiation damage is suggested by previous studies on the effects of total body irradiation in the histamine depleted rats (van den Brenk, 1957) and is supported by our finding of increased local damage in such depleted rats. It once more raises the question of the essential role of histamine in animal physiology. Perry (1956) has drawn attention to the high histamine and mast cell content of rat and mouse tissues in comparison with certain other mammals—a concentration of 40 μg. histamine per gram of skin in the rat, compared with 3–8 μg./gm. in guinea-pig and man. He records that Compound 48/80 causes little reduction in histamine content of guinea-pig skin. It causes a marked reduction of histamine in rat skin, but a non-releasable residue remains apparently not contained by mast cells which approximates in amount (3 μg./gm.) to that in guinea-pig skin. He points out the difference in behaviour of rat and guinea-pig skin to active and passive skin allergies and suggests that the high content of mast cells with their releasable histamine in rats and mice may be a defence mechanism. These creatures react to a foreign antigen, not so much by the production of a specific antibody, but rather by active phagocytosis in conjunction with tissue dilution of the foreign substance brought about by the capacity to produce large amounts of histamine. Perry's theory warrants consideration, but in attempting to draw conclusions solely from rats and mice as experimental animals, it confuses the whole issue of histamine release agents in relation to human physiology. If Perry's theory is valid, it is not surprising that cell damage induced in rats by irradiation or other means cannot be so satisfactorily resolved in the presence of histamine depleted tissues.

SUMMARY

The results of experiments on rats are recorded, dealing with the local effect of X-irradiation on skin, abdominal tissues, histamine release and mast cell changes.

The morphological changes in mast cells which follow irradiation, differ from those induced by a histamine depleting agent (Compound 48/80).

The mast cells of rats subjected to a high dose of X-irradiation (4000 r) respond to intraperitoneal injections of Compound 48/80, in a normal manner.

Rats subjected to an LD100/5 days of 2000 r total body irradiation, respond to intraperitoneal injections of Compound 48/80 by severe shock symptoms.

Rat skin irradiated with a single tissue dose of 4000 r gives essentially normal reactions to intradermal injections of histamine, Compound 48/80, croton oil and a second irradiation dose of 10,000 r.

Irradiation of skin in histamine depleted animals causes local erythema and tissue damage earlier and of greater intensity than similar irradiation of skin in undepleted control animals.

The significance of these findings are discussed in relation to the hypothesis that histamine release plays a significant role in radiation damage. The evidence presented fails to support this view.

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RELATIONSHIP OF AGAR DIFFUSION PATTERNS TO AGGLUTINATING AND PROTECTIVE PROPERTIES OF *BRUCELLA SUIS* ANTISERA

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OLITZKI AND SULITZEANU (1957, 1958) examined with the aid of the technique of Ouchterlony (1948) the antigen-antibody reaction between *Brucella suis* hyperimmune sera and their corresponding antigens present in acetone-killed bacteria or sonic extracts.

They noted the appearance of at least 6 and with certain immune sera even 9 precipitation lines. The question arose whether any of these antigenic components are responsible for the agglutination of intact bacteria by the corresponding immune sera. It was also of interest to determine whether any of these components participate in the bactericidal reaction mediated *in vivo* by the immune sera.

It is generally accepted (Elberg and Silverman, 1949) that in Brucellosis agglutination titres are not correlated with the ability to withstand infection. This would suggest that agglutinins are distinct from what could be called " protective antibodies ". In the present work an attempt was made to determine to what extent the 2 activities could be separated.

Br. suis was chosen for this study because of the ease with which strongly precipitating antisera could be obtained against it. Although interest centres at present on *Br. abortus* which is the organism employed for the preparation of living attenuated vaccines in cattle, it was felt that this preliminary work with *Br. suis* might not be altogether out of place, in view of the close antigenic relationship between the two organisms.

The general plan of the work was as follows : bacteria or their extracts were subjected to various treatments intended to destroy some of their antigenic components. They were then used to absorb the immune serum prepared against intact bacteria. Sera were thus obtained containing antibodies against fewer antigenic components, as demonstrated by the Ouchterlony agar gel diffusion technique. These sera were tested for their agglutinating power and for their ability to protect mice against *Br. suis* infection. In addition, a few sera were prepared against bacteria previously subjected to various treatments, and tested in the same manner.

MATERIALS AND METHODS

The experimental procedures have been described in the foregoing paper (Olitzki and Sulitzeanu, 1958). A few additional details are given below :

Antisera

The following antisera were tested:

RS: reference serum. A pooled serum obtained from several rabbits after prolonged immunization (3–4 months) with the acetone killed strain 39 of *Br. suis*.

Serum 24: antiserum to *Br. suis* 39 killed by acetone and heated at 60°.

Serum 25: antiserum to *Br. suis* 39 killed by acetone and treated with 0·05 N NaOH.

Serum 46: antiserum to *Br. suis* 39 killed by acetone and treated with N HCl.

Serum 48: antiserum to *Br. suis* 39 killed by acetone and treated with 0·1 N HCl.

Details on the preparation of the variously treated antigens mentioned above have been given (Olitzki and Sulitzeanu, 1958).

Agglutination tests

Preliminary experiments showed that titres were always higher with *Br. abortus* 19 than with the homologous organisms. Tests were therefore performed with *Br. abortus* 19. 100 per cent agglutination was taken as end-point, but when greater accuracy was desired, 50 per cent end-points were used. The technique of the agglutination test has been described (Sulitzeanu, 1955).

Protection tests

The protection test used was a modification of the test described by Sulitzeanu (1955) for *Br. abortus*. 0·1 ml. of a 1/100 dilution of serum in phosphate buffered saline (pH 7·2) were injected into the thigh muscles of the left hind leg of each of a group of five mice. The next day 3×10^4 *Br. suis* 39 were administered intra-abdominally to all protected mice and to an additional untreated group. Seven days later the mice were killed with ether and the spleens removed. Each spleen was triturated with sterile glass powder in a mortar. The smooth suspension obtained was suspended in 10 ml. broth and 0·2 ml. of the suspension were spread on each of two Trypticase Soy agar plates. Colonies were counted after 3 days' incubation. The results were expressed as Protection Index (P.I.).

P.I. = Mean log. of plate count of control mice − mean log. of plate count of immune mice.

In the original test with *Br. abortus*, the spleens of each treatment group were triturated together. However, in the test with *Br. suis* it was necessary to count each spleen separately, owing to the variation in the susceptibility of the mice to infection with this organism. Geometric means were used in the calculation of the results since in such cases they give a more adequate picture of the true distribution of counts than the arithmetic means.

Preparation of ultracentrifuged sonic extract (USE)

One gram of acetone-killed organisms was disintegrated in a Raytheon magnetostriction oscillator. The suspension obtained was centrifuged at 10,000 r.p.m. for 1 hr., the sediment suspended in a small volume of saline and subjected to a further disintegration. This procedure was repeated 5 times, to ensure as complete a destruction of the whole bacteria as possible. The supernatant fluids were pooled and centrifuged in a Spinco ultracentrifuge for 30 min. at 40,000 r.p.m. 40 ml. of a crystal clear solution were obtained, containing approximately 2 mg. protein per ml. It was stored in the refrigerator at 4°, with merthiolate 1 : 10,000 as preservative. The solution did not keep well, a heavy precipitate forming during a few days of storage. The precipitate could, however, be removed by centrifugation, without apparent loss of activity. The agar diffusion pattern of this preparation was practically identical with that of the original sonic extract (Fig. 5).

Preparation of sediment of ultracentrifuged sonic extract (SUSE)

The sediment left from the preparation of USE was subjected to several cycles of centrifugation to remove the intact bacteria. It was suspended in saline and centrifuged for 30 min. at 3000 r.p.m. in an MSE Superminor centrifuge. The supernatant fluid was removed and subjected to a similar treatment. After repeating the procedure 4–5 times, an opalescent suspension of insoluble amorphous material was obtained. It stained Gram negative and was devoid of whole bacteria. From the sediments left after the above centrifugation, more material could be isolated by resuspending in saline and repeating the whole procedure. The material was finally concentrated by centrifugation for 20 min. at 8000 r.p.m. and resuspending in a small volume of saline. This suspension contained 0·9 mg. dry material per ml. Generally

no lines, or at most a single weak line, were obtained with this preparation when tested in agar against RS (Fig. 5).

RESULTS

Agglutination titres of Br. suis *antisera, with* Br. abortus 19 *and* Br. suis 39 *as antigens* (acetone-killed bacteria)

A comparison of the two organisms as antigens in the agglutination test is shown in Table I. Titres with *Br. abortus*, were always higher, although the concentration of the 2 antigens was identical.

TABLE I.—*Agglutination Tests with* Br. abortus *and* Br. suis

Serum No.	Description of serum	Reciprocals of agglutinin titres with antigen	
		Br. abortus 19	*Br. suis* 39
9	RS absorbed extract treated with 0·05 N HCl	160	40
19	RS absorbed extract heated at 60°	300	100
26	RS absorbed extract treated with NaOH	300	100
RS	Reference serum	640	320
1	Antiserum to *Br. suis* incorporated in Freund's adjuvant*	640	160
2	Antiserum to *Br. suis* incorporated in Freund's adjuvant	2560	640
25	Antiserum to *Br. suis* killed with acetone and treated with NaOH	320	40
24	Antiserum to *Br. suis* heated at 60°	1280	160

* The agar diffusion pattern of this serum is shown in Fig. 4.

Diffusion pattern of RS

The diffusion pattern of this serum has been described (Olitzki and Sulitzeanu, 1958). Briefly, it gave 7 lines, numbered 1–7 starting from the antigen source. Of these, lines 3–7 were easily reproducible, whilst 1 and 2 failed sometimes to appear. Attention was therefore concentrated on the former.

Protection tests

The type of results obtained are illustrated by Table II, which also gives an idea of the variability of counts obtained with mice belonging to the same treatment group. Because of this variation the protection, although significant, was generally of a low order, with mean differences of 1–1·5 log. between the immune and the non-immune group.

TABLE II.—*Protective Power of Two* Br. suis *Antisera Against Infection by* Br. suis

Mouse No.	Mean plate count of spleens of mice protected with		
	Serum 29	Serum 20	No serum
1	1	83	44
2	1	400	100
3	6	420	320
4	24	540	1100
5	440	580	1450
Mean log. count	1·2	2·53	2·47
Protection index	1·27	0	—

TABLE III.—*Details of Sera Tested*

Serum No.	Description
RS	Reference serum.
8	RS absorbed sonic extract treated with 0·3N HCl.
10	RS absorbed bacteria treated 0·1 N HCl.
12	RS absorbed bacteria treated N HCl.
20	RS absorbed untreated acetone-killed bacteria.
22	RS absorbed bacteria treated with 0·05 N NaOH.
24	Antiserum to bacteria heated at 60°
25	Antiserum to bacteria treated with 0·05 N NaOH.
26	RS absorbed sonic extract treated with 0·05 N NaOH.
27	RS absorbed bacteria heated at 100°.
29	RS absorbed sonic extract heated at 60°.
46	Antiserum to bacteria treated N HCl.
48	Antiserum to bacteria treated with N/10 HCl.
49	RS absorbed with SUSE.
50	RS absorbed with USE.

Diffusion pattern in relation to agglutinating and protective properties

The results of the diffusion tests are summarized in column 2 of Table IV. As a result of absorptions, sera were obtained of widely different antibody content. Their agglutination titre is given in column 3. All absorptions are seen to have caused a reduction in agglutinin titre, showing that the antigens concerned were fairly stable and did not deteriorate in spite of the drastic treatment sometimes

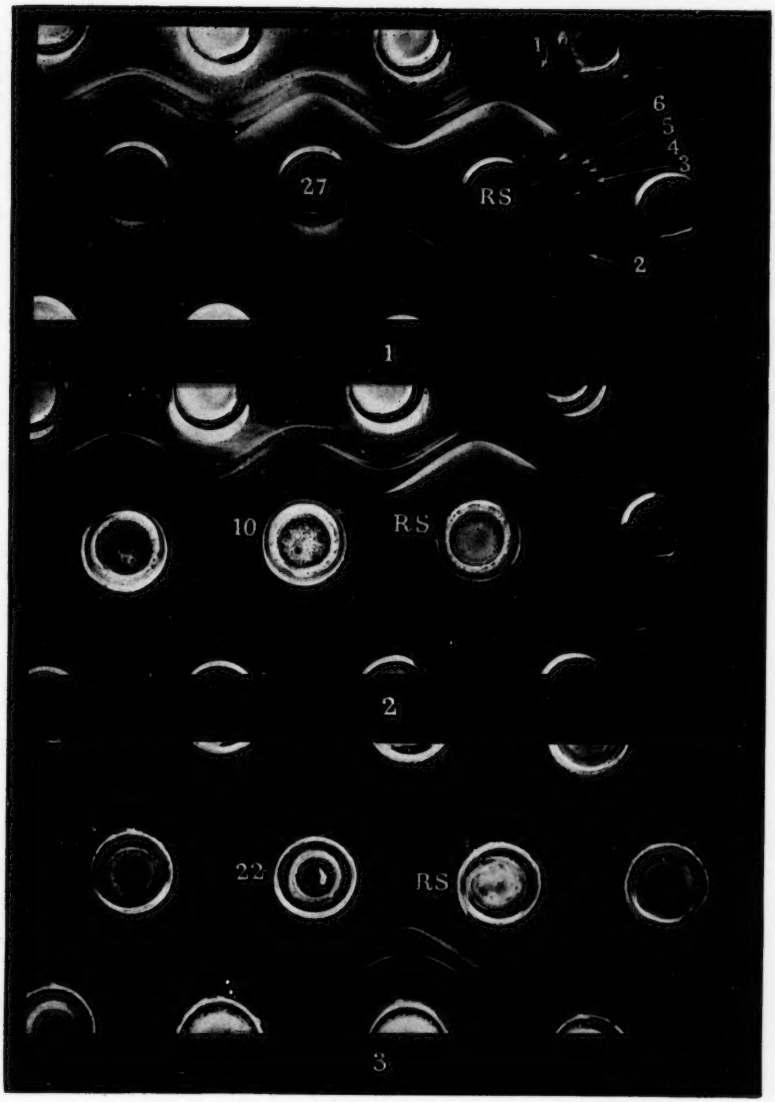
TABLE IV.—*Diffusion Pattern, Agglutinating Titres and Protection Indices of* Br. suis *Antisera*

Serum No.	Diffusion lines given by serum					Agglutinin titre*	P.I.
	III	IV	V	VI	VII		
RS	+	+	+	+	+	900	2·23
8	+	+	+	+	+	550	2·13
22	+	+	+	+	±	20	0
27	+	+	+	±	—	0	0
10	+	+	+	±	±	0	0·32
12	+	+	+	—	±	0	1·12
20	—	+	+	±	—	0	0
29	+	—	—	±	—	300	1·27
25	—	+	+	—	—	300	2·17
26	+	—	—	—	—	300	1·46
48	—	—	—	±	—	700	1·11
24	—	—	—	—	—	900	1·00
46	—	—	—	—	—	700	1·44
49	+	+	+	+	+	0	0
50	—	—	—	—	—	160	1·44

* Titres expressed as 50 per cent end-points.
\+ Strong line.
± Weak line.

EXPLANATION OF PLATES

FIG. 1.—Diffusion patterns of RS (agglutination titre 1/900) and serum 27 (agglutination titre 0).
FIG. 2.—Diffusion patterns of RS and serum 10 (agglutination titre 0).
FIG. 3.—Diffusion patterns of RS and serum 22 (agglutination titre 1/20).
FIG. 4.—Diffusion patterns of RS and serum 1 (prepared against bacteria incorporated in Freund's adjuvant—agglutination titre 1/640).
FIG. 5.—Diffusion patterns of sonic extracts (SE), ultracentrifuged sonic extracts (USE) and sediment of ultracentrifuged sonic extract (SUSE).



Sulitzeanu.

applied. In contrast, several treatments caused modifications in the bacteria or extracts, resulting in inability to absorb some of the precipitating antibodies (sera 8, 22, 27).

No correlation can be seen between the diffusion patterns and agglutination titres. Sera showing little change in the diffusion pattern (10, 12, 22, 27) lost most or all agglutinins, whilst other sera lacking several (25, 26) or all diffusion lines (24) had marked to strong agglutinating properties (see plates).

The lack of correlation between agglutinin titres and diffusion pattern indicated that the antigens and antibodies involved in agglutination took little or no part in the production of lines. This was confirmed by the finding of sera of the type of 24 and 46, which had the same agglutinin titre as RS, but did not produce any line whatsoever, Several explanations could be offered for this phenomenon :

The agglutinogens and agglutinins are found in concentrations below those required to give a diffusion line.

The agglutinogens are bound to insoluble cellular material and unable, therefore, to diffuse and give a precipitation line.

There may be several agglutinogens, which do not produce lines because of either reasons.

The second explanation seemed more likely and the experiments which follow were undertaken in an effort to test it.

Absorption of agglutinins with USE

To tubes containing 0·1 ml. quantities of RS, varying quantities of USE were added. The volume was brought to 1 ml. with saline and the tubes treated as described in the previous paper under " absorption of sera ". After removing the precipitates by centrifugation, aliquots from each tube were tested for antigen excess by addition of 1 drop RS (" antigen " refers here to the complex antigenic mixture found in USE). Other aliquots were titrated for agglutinin content.

Table V shows that the agglutinin titre of RS could not be reduced below 1/80 by absorption with USE, even when a large excess of antigen was added. On the

TABLE V.—*Agglutinin Titres of RS after Absorption with Varying Quantities of USE*

USE added for absorption (mg. protein)	AG excess	Reciprocals of agglutinin titres of absorbed serum									
		15	30	40	60	80	120	160	320	640	1280
None	—	+	+	+	+	+	+	+	+	+	—
0·006	—	+	+	+	+	+	+	+	+	+	—
0·02	+	+	+	+	+	+	+	+	+	±	—
0·06	+	+	+	+	+	+	+	+	+	—	—
0·12	+	+	+	+	+	+	+	+	+	—	—
0·2	+	+	+	+	+	+	+	+	—	—	—
0·4	+	+	+	+	+	+	—	—	—	—	—
0·6	+	+	+	+	+	+	—	—	—	—	—
0·8	+	+	+	+	+	+	±	—	—	—	—
1	+	+	+	+	+	+	±	—	—	—	—
1·2	+	+	+	+	+	+	+	—	—	—	—
1·6	+	+	+	+	+	+	+	—	—	—	—
1·8	+	+	+	+	+	+	+	—	—	—	—
3	+	+	+	+	+	+	+	+	—	—	—
4·2	+	—	+	+	+	+	+	+	—	—	—

contrary, increasing the concentration of the antigen seemed to increase somewhat the titre of the absorbed serum.

A similar experiment was performed with three other sera, Nos. 24, 25, 2. The amounts of USE used in absorptions were 0·1, 0·4, 2, 3·8 mg. protein per 0·1 ml. serum. The tubes to which 0·1 mg. antigen had been added showed little or no antigen excess after absorption. All the others had marked antigen excess. The agglutinin titres of the absorbed sera are summarized in Table VI. Their behaviour was identical to that of RS, *i.e.*, incomplete absorption of agglutinins by USE and slight increase of agglutinin titre with increase of amount of antigen.

TABLE VI.—*Agglutinin Absorption Tests with USE*

Serum No.	Amount USE used in absorption (mg. protein)	Reciprocals of agglutinin titres of supernatant fluids						
		20	40	80	160	320	640	1280
2	None	+	+	+	+	+	+	—
	0·1	+	+	+	+	+	+	—
	0·4	+	+	+	+	+	+	—
	2	+	+	+	+	—	—	—
	3·8	+	+	+	+	+	—	—
24	None	+	+	+	+	+	+	—
	0·1	+	+	+	+	+	—	—
	0·4	+	+	+	+	—	—	—
	2	+	+	+	+	—	—	—
	3·8	+	+	+	+	+	—	—
25	None	+	+	+	+	—	—	—
	0·1	+	+	+	—	—	—	—
	0·4	+	+	+	—	—	—	—
	2	+	+	+	+	—	—	—
	3·8	+	+	+	+	—	—	—

Absorption of RS with SUSE

To 0·1 ml. of RS diluted 1 : 1·5, varying quantities of SUSE were added. The volume of each tube was brought to 1 ml. with saline. After the usual absorption procedure the supernatants were titrated for agglutinin content. Table VII

TABLE VII.—*Agglutinin Titres of RS after Absorption with Varying Quantities of SUSE*

Amount SUSE used in absorption (mg. dry material)	Reciprocals of agglutinin titres of supernatant fluids						
	15	30	60	120	240	480	960
None	+	+	+	+	+	+	—
0·02	+	+	+	+	+	+	—
0·06	+	+	+	+	+	+	—
0·11	+	+	+	+	±	—	—
0·15	+	+	+	+	—	—	—
0·20	+	+	+	—	—	—	—
0·25	+	+	—	—	—	—	—
0·3	+	+	—	—	—	—	—
0·5	+	—	—	—	—	—	—
0·8	±	—	—	—	—	—	—
1·2	±	—	—	—	—	—	—

shows that SUSE was more efficient in reducing the agglutinin titre of RS than USE. The former brought the titre down to below 1/15, whilst the latter could not reduce the titre to below 1/80.

In order to compare the efficiency of SUSE with that of whole bacteria, an absorption experiment was set up with acetone-killed bacteria. To 0·1 ml. quantities of RS various quantities of a suspension containing 20 mg./ml. acetone-killed organisms were added. The absorbed sera were titrated for agglutinin content and the results, given in Table VIII, show that 1 mg. bacteria brought the titre down to 1/40, whilst 1·2 mg. were sufficient to reduce it below this figure. It can be seen that whilst the serum absorbed with 0·7 mg. whole bacteria still gave a slight reaction at 1/80 dilution, that absorbed with 0·25 mg. SUSE was negative at 1/60. Weight per weight SUSE was therefore more efficient in absorbing agglutinins than whole bacteria.

TABLE VIII.—*Absorption of RS with Acetone-killed Whole Bacteria*

Mg. bacteria used in absorption	Reciprocals of agglutinin titres			
	40	80	160	320
0·5	+	+	+	—
0·7	+	±	—	—
1	+	—	—	—
1·2	—	—	—	—

Agglutinating versus protecting power

Column 4 of Table IV summarizes the results of the protection tests. As already mentioned, the protection was of a low order. If, however, we take a P.I. of 1 (corresponding to a mean level of infection in protected mice 10 times as low as that in the non-protected mice) as indicative of protection, then practically all sera capable of agglutinating bacteria to any titre, were also able to protect mice against infection (serum 12 was the only exception). Sera from which the agglutinins had been removed were devoid of protective power. These results indicate that agglutinating and protecting antibodies may be identical.

DISCUSSION

Several points of interest have emerged from this investigation. Somewhat unexpectedly, no correlation could be found between the agglutinin titre of *Br. suis* antiserum and its diffusion pattern in agar gel. The loss of any of the 7 diffusion lines was not correlated with loss of agglutinating power and the presence of agglutinating antibodies was not correlated with ability to produce lines in agar.

Since the agglutinins could be removed by absorption with whole bacteria, it was inferred that the agglutinogens would be situated on the bacterial surface. And since a variety of treatments left the agglutinin absorbing power intact, it could be deduced that the agglutinogens would be bound to some sort of insoluble cell material. The failure of the agglutinogen-agglutinin system to produce lines in agar strengthened this assumption. This was found indeed to be the case, as insoluble cell debris (SUSE) were shown to absorb the agglutinins more efficiently than whole bacteria. On the other hand, the ultracentrifuged sonic extract could

also absorb most, although not all, agglutinins. It appears, therefore, that two agglutinogens might be involved, both of which are present in SUSE, but only one present in USE. Whether the agglutinogen found in soluble form in USE is a low molecular breakdown product, derived from the insoluble material by sonic disintegration, cannot be ascertained at present.

Of particular interest was the finding that both agglutinating and protecting properties of the serum could be removed by absorption with SUSE, an insoluble material which either gave no diffusion lines at all or produced a very weak line only. This material is probably the cell wall or part of it, as proved by the fact that whole bacteria are equally able to absorb the 2 properties.

The immunizing efficiency of cell wall material has been proved with *Pasteurella tularensis* by Shepard and co-workers (1955) and it is likely that *Brucella* cell wall plays a similar part. This fact seems to have escaped attention so far, in spite of the large amount of work already done on immunizing fractions of *Brucellae* (reviewed by Elberg and Silverman, 1949) since all efforts were directed towards isolation of soluble fractions. Further work is in progress in this laboratory on the chemical and immunological characterization of *Brucella* insoluble cell material. A very good correlation was found between the ability of the serum to agglutinate *Brucella* organisms and its ability to protect mice passively against *Brucella* infection. Although the protection test with *Br. suis* was less satisfactory than that with *Br. abortus*, owing to the variability in the susceptibility of mice to infection with this micro-organism, the almost unfailing correlation of the 2 properties makes it highly probable that both protection and agglutination are due to the same antibodies.

It is worth mentioning that, whilst the agglutinins appeared in the circulation of rabbits fairly quickly, reaching a maximum at 14-30 days, the precipitating antibodies involved in the production of agar diffusion lines required, as a rule, a prolonged period of intensive immunization, before easily visible lines could be obtained. There was no increase in agglutinin titre during this time. The failure of the agglutinating antibodies to rise in titre might well be a case of antigenic competition, of which several examples have been mentioned in the literature (Barr and Llewellyn-Jones, 1953 ; Topley and Wilson, 1955). If, as we believe, the agglutinating antibodies are identical with protective antibodies and play therefore a role in the defence mechanisms, this competition might be of practical importance in immunization of animals and it deserves further study.

SUMMARY

By a variety of absorption and immunization procedures *Br. suis* antisera were obtained of widely different agar diffusion patterns. No one of the 7 lines present could be correlated with the presence or absence of agglutinins.

Good correlation was found between agglutinating and passive protection power of sera. The two activities seem to be due to the same antibodies.

Two agglutinogens were demonstrated in the insoluble cell debris of *Br. suis*. One of them was also found in soluble form after sonic disintegration of bacteria.

The possible role of antigenic competition between the immunizing and inert antigens has been discussed.

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FRACTURE REPAIR IN THE RAT DURING AMINOACETONITRILE ADMINISTRATION AND FOLLOWING ITS WITHDRAWAL

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THE production of osteolathyrism and aortic aneurysms by certain aminonitriles is now well established and evidence has accumulated that these changes are associated with alterations in the intercellular substance of connective tissues. Selye (1957*c*), reviewing the literature on lathyrism, has pointed out that the severity and site of the lathyrous lesion has been shown to depend on various factors such as age, hormonal state and muscular stress. The most rapid and gross changes occur in young animals but it has become clear that the lathyrous factor is most effective during tissue growth, not only in the growing animal but also during phases of rapid growth in older animals. Thus, in the adult, any state in which there is proliferation of connective tissue, usually in the form of a granulation tissue, forms a suitable medium to study the effects of the active principles and, for bone changes, healing fractures are especially appropriate.

That the mechanical properties of connective tissues are altered following the ingestion of sweet pea (*Lathyrus odoratus*) seeds is beyond dispute ; slipping of epiphyses and rupture of aortic aneurysms show this clearly (Ponseti and Shepard, 1954). Since the demonstration of the active principle β-(N-γ-L-glutamyl)-aminopropionitrile (Schilling and Strong, 1954) in these circumstances, this substance and its analogues, of which aminoacetonitrile (AAN) is one of the most active, have been used frequently for experimental purposes. Enzinger and Warner (1957) have shown that, in healing wounds in AAN-treated rats, there is a reduction in tensile strength which returns rapidly to normal when the AAN is withdrawn. The walls of granuloma pouches formed during AAN administration are weaker than usual and collapse (Mielke, Lalich and Angevine, 1957). The effect of AAN in adult animals is slight, except for the formation of periosteal " osteomas " which depend for their development on muscle tension (Hamre and Yeager, 1957) since, when muscle attachments to the bone are severed, no periosteal proliferation takes place at that site. A detailed study of the formation of the wall of sterile turpentine abscesses has shown that the maturation of collagen is delayed (Hurley, Storey and Ham, 1958).

Whether the production of soft connective tissue in the AAN-treated animal is due to some change in ground substance or a deficiency of collagen formation is not yet certain. Menzies and Mills (1957) are of the opinion that there is an excessive accumulation of intercellular ground substance in both aorta and bone of lathyrous rats, which is in agreement with observations of Churchill, Gelfant, Lalich and Angevine (1955) who described increases in polysaccharides in the aortae of rats fed sweet pea seeds. On the other hand Ponseti, Wawzonek, Shepard,

Evans and Stearns (1956) have shown that the radioactive sulphur uptake in the aorta is the same in both normal and lathyrous animals and the preliminary observations of Dasler (1957) also indicate that there is no deficiency in mucopolysaccharide formation.

Regarding the later stages of connective tissue formation, on the one hand no difference was found in collagen content, of femora (Dasler, 1954) nor change in electron microscopic appearance of rat tail tendons (Dasler, 1957) but, by contrast, there is a decrease in the amount of hydroxyproline in the wall of croton oil pouches in β-aminopropionitrile (BAPN) treated rats, where microscopic appearances suggesting delayed collagen synthesis have been observed (Mielke *et al.*, 1957).

It is thus clear that the defect in connective tissue has not yet been finally elucidated, but detailed histological study shows that during the formation of abscess walls there is a delay in maturation of collagen. The initial production of collagen fibrils occurs normally but during the maturation process amorphous material accumulates between fibrils (Hurley *et al.*, 1958).

The present paper is concerned with a detailed study of the repair of experimental fractures of the tibia of the rat. Selye (1957*b*) showed that giant callus formation occurred in AAN-treated rats during the repair process, but owing to excessive mobility of the bones their dissection from surrounding muscle was difficult. For this reason he discontinued the AAN administration after 12 days' treatment and allowed the callus to consolidate before examining it 5 days later. This procedure could lead to some confusion as it is not known whether the final changes studied were due to the effect of AAN or to its withdrawal. The sudden withdrawal of hormones and other substances can lead to reversal effects which complicate the experimental results; this has been shown recently with respect to the administration and withdrawal of cortisone (Storey, 1957; Storey, 1958) and emphasizes the point that such studies should be correlated at various time intervals during and after any progressing pathological change.

MATERIALS AND METHODS

Thirty female rats (Sprague-Dawley and Wistar) weighing 90–110 g. were used. They were fed on a diet of Baristoc pellet—a local proprietary food—supplemented by fresh green vegetables and water *ad libitum*. The animals were divided into three groups and every second day one animal from each group was killed until the end of the experiment on the twentieth day. Those in the first group were used for the normal study; in group 2 five mg. AAN (prepared by the method of Anslow and King, 1941) was injected twice daily subcutaneously into the inner side of the thigh. In the third group AAN was injected at the same dose level until the twelfth day when it was discontinued, one animal being killed every second day until the twentieth day as in the other groups.

In all groups the left tibia was fractured as described by Selye (1957*b*). At the times stated, the animals were killed by ether anaesthesia and both the right and left tibiae dissected from the animal. Where possible the muscles were dissected from the bones but in the early stages of fracture repair, particularly in the AAN-treated animal, a considerable amount of muscle was left *in situ* in order to hold the ends of the fractured bones together. Most specimens were fixed in neutral formal-saline, a few being fixed in Bouin's solution. Decalcification was carried out in 7½ per cent nitric acid; then the tissues were washed in sodium sulphate for one day and finally washed in running water for a further 24 hr. The specimens were embedded in paraffin wax in the usual way; sections were cut at 5–7 μ.

The following stains were used: Ehrlich's haematoxylin and eosin, Van Gieson, Alcian blue (Steedman, 1950), periodic acid Schiff-PAS (McManus, 1948), ammoniacal silver (Gomori as modified by Lillie, 1948), thionin-phosphotungstic acid (Schmorl, 1948).

RESULTS

Macroscopic changes

The AAN fractures were more mobile than normal from 6–8 days onwards and on dissection the callus was larger and softer. By 20 days the fractured legs of AAN-treated animals were grossly larger than controls due to voluminous callus and, as the tibia was bent at an angle of 30° more than the control animal at the fracture site, considerably shorter than the opposite leg. The animals, where

EXPLANATION OF PLATES

FIG. 1.—Photographs of fractured tibiae showing the callus at 20 days cut in section. Normal tibia on the left, continuous administration of AAN in the centre showing the formation of a giant callus and shortening of the leg due to increased angulation of the fractured bone. The specimen on the right shows the effect of withdrawal of AAN after 12 days' administration when a well-formed giant callus is seen.

FIG. 2–5.—Photomicrographs of collagen forming between the callus on the anterior aspect of the tibia. Silver impregnation. × 333.

FIG. 2.—Normal rat at 12 days showing formation of dense collagen.

FIG. 3.—AAN-treated rat at 12 days showing formation of fine agyrophilic fibrils instead of dense collagen seen in Fig. 2.

FIG. 4.—AAN withdrawn at 20 days. Dense interweaving bundles of mature collagen.

FIG. 5.—AAN-treated rat at 20 days showing immature connective tissue fibres poorly stained with silver with only a few dense bundles of collagen running through it.

FIG. 6–11.—Photomicrographs of callus forming near the fracture site. PAS, haematoxylin. × 27.

Photomicrographs taken with Ilford Panchromatic film.

FIG. 6.—Normal rat at 4 days, bone forming from periosteal tissues.

FIG. 7.—AAN rat at 4 days, greatly enlarged cellular periosteum with bone matrix formation just starting next to the shaft of the tibia.

FIG. 8.—Normal rat at 12 days showing a well-formed bony callus where remodelling processes are in progress.

FIG. 9.—AAN-treated rat at 12 days, the callus is greatly enlarged, bone is still forming rapidly from connective tissue which is not yet completely stained with PAS.

FIG. 10.—Normal rat at 20 days. The callus is no larger than at 12 days, remodelling process has replaced most of original bone, marrow largely composed of fat cells.

FIG. 11.—AAN rat at 20 days. Callus now relatively inactive, most of the original bone has been removed but not replaced during the remodelling process. The marrow is still cellular and active.

FIG. 12–17.—Photomicrographs of the site of union at the posterior aspect of the fracture at 20 days, and the corresponding epiphysis. PAS, haematoxylin. × 27.

FIG. 12.—Normal rat showing where union has taken place by cartilage.

FIG. 13.—Normal rat showing a narrow epiphysis.

FIG. 14.—AAN-treated rat. The callus is widely separated by loose connective tissue and most of the adjoining cartilage has been removed with little replacement by bone.

FIG. 15.—AAN-treated rat. Grossly widened epiphyseal cartilage consisting of islands of swollen chondrocytes in a distorted matrix.

FIG. 16.—AAN withdrawn after 12 days' administration. Callus is separated by dense connective tissue, some cartilage still remains and is being replaced by dense bone.

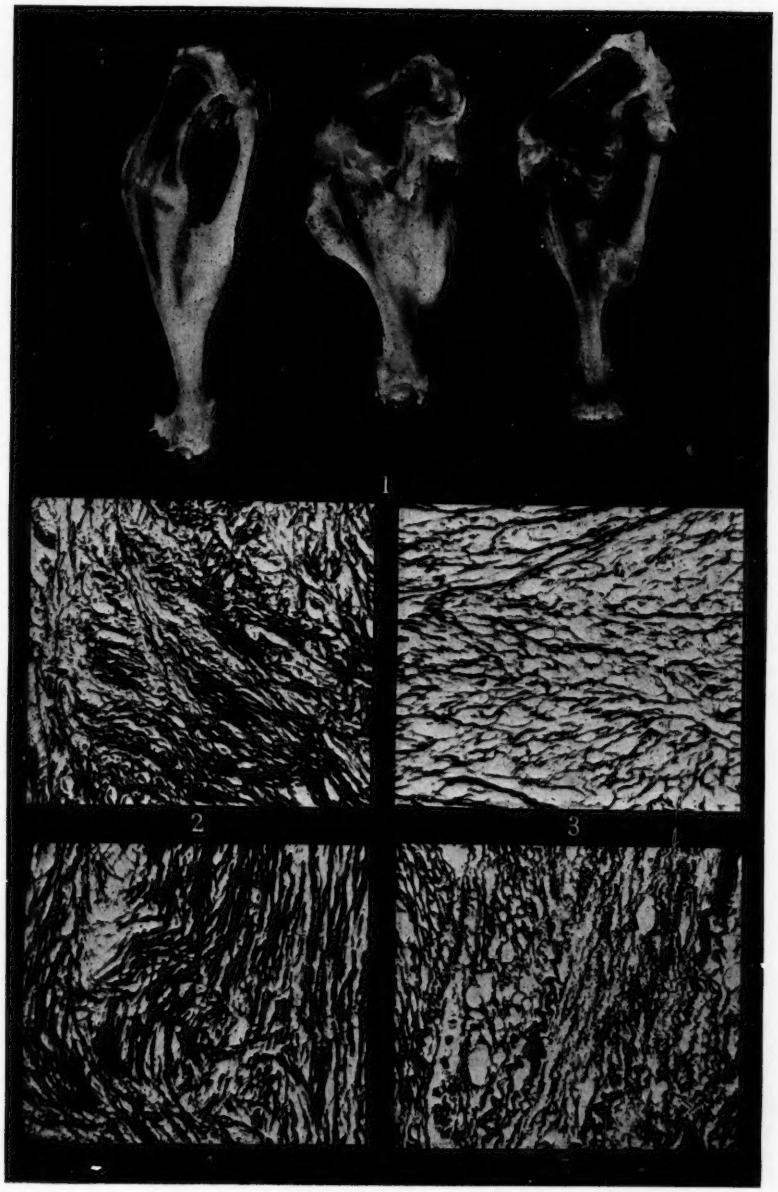
FIG. 17.—AAN withdrawn after 12 days' administration. Epiphyseal cartilage has returned to near normal, metaphyseal trabeculae are shorter and distorted.

FIG. 18.—Normal rat 20 days after fracture showing deeply PAS positive bone in the centre, and above and below lightly stained more mature bone with fewer cells formed during remodelling phase. × 350.

FIG. 19.—AAN-treated rat 20 days after fracture showing granular appearance of deeply staining PAS positive bone and absence of granules in more lightly staining bone formed during remodelling process. × 350.

FIG. 20.—AAN-treated rat 20 days after fracture showing large globules of PAS positive material accumulating around an osteocyte (lower left) at edge of newly forming bone. PAS. × 1400.

FIG. 21.—AAN withdrawn at 12 days, 20 days after fracture showing a reduction in size of globules. PAS. × 1400.



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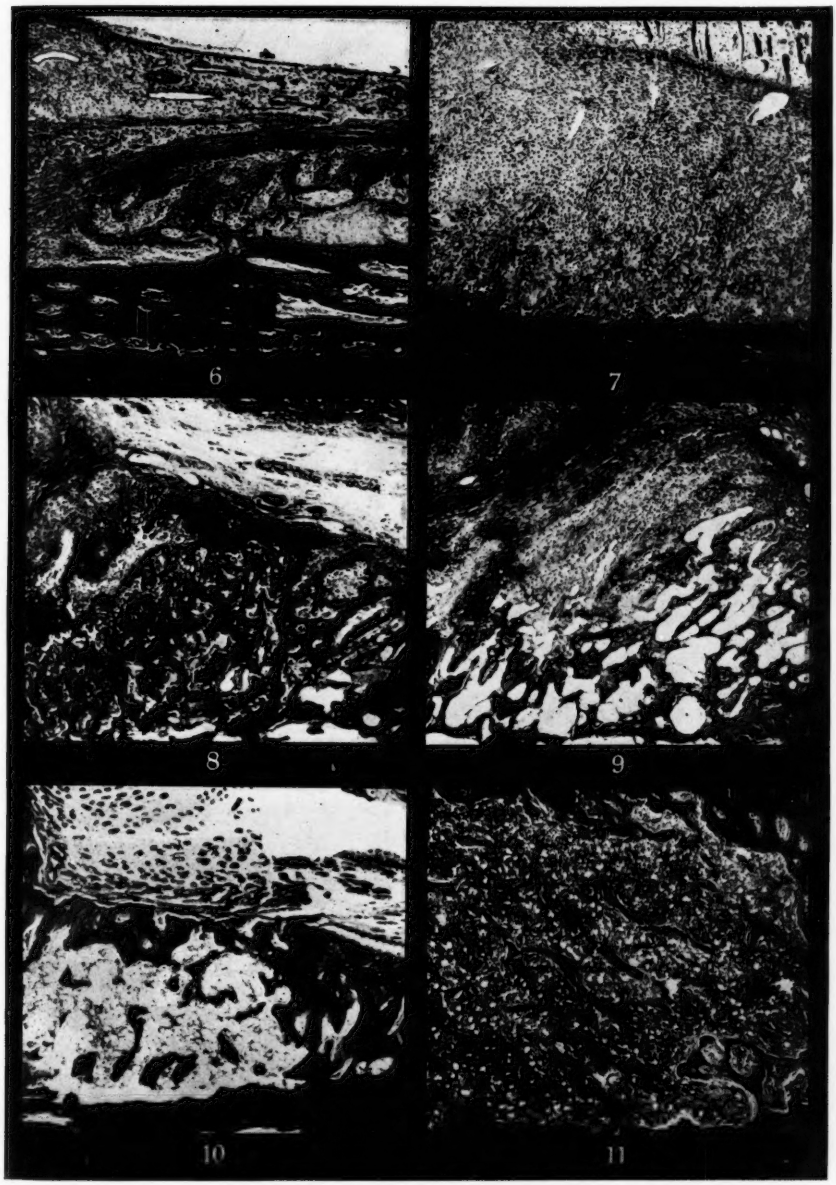

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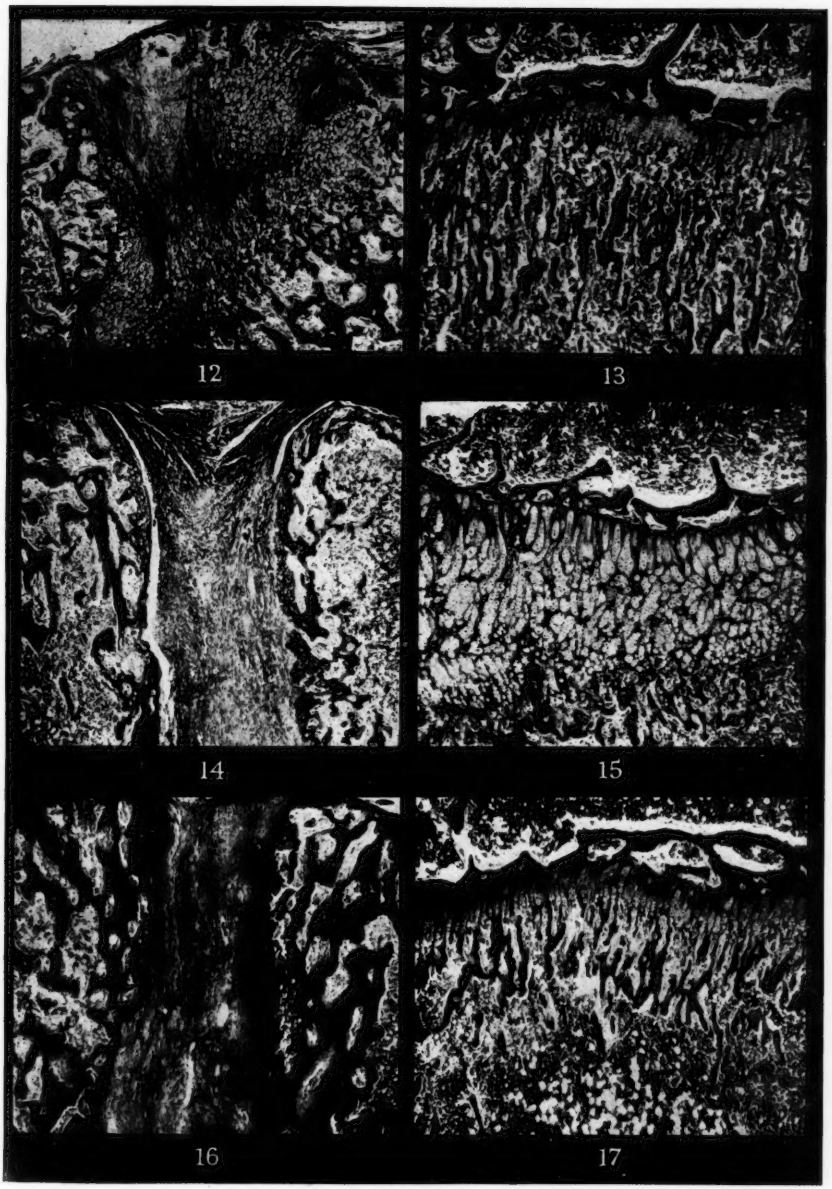

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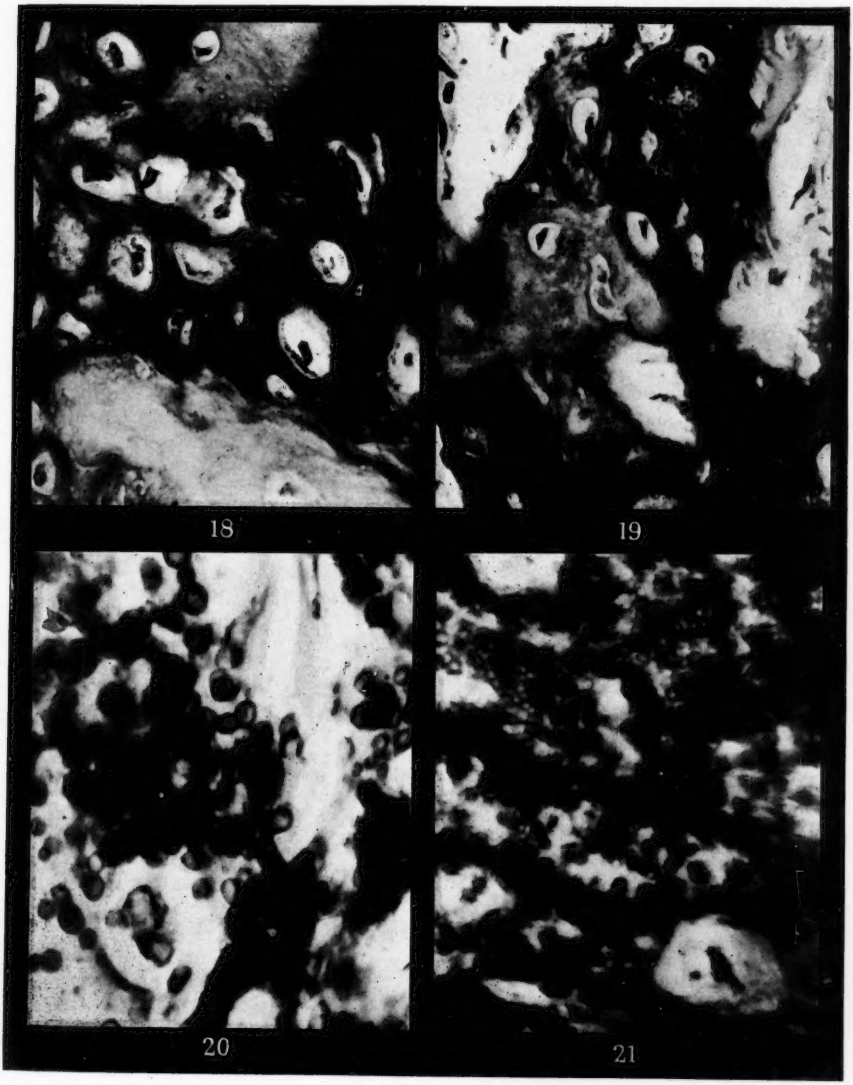

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20 21

Storey and Varasdi.

AAN had been withdrawn, had smaller and less mobile callus, the angle of the two parts of the fractured tibia being more obtuse than in the animal treated continuously with AAN. This can be seen in Fig. 1. There was some difficulty in dissecting the leg from muscle in the continuously treated animal but not in the normal or " withdrawn " one. When the callus was fixed in formalin the specimen was bisected prior to sectioning and in the AAN animal only a thin shell of bone was visible around the periphery of the callus ; there was considerably more bone present in the animal where AAN had been withdrawn. The articulation of the fibula on the tibia was enlarged in the fractured limb, but in the animals treated with AAN either for part or the whole of the period up to examination (Groups 2 and 3) it was grossly increased in size.

Microscopic changes

Normal Fracture

At the end of the first 2 days the area around the fracture site can be seen to be swollen and a haematoma separates the two bones at the fracture. The periosteum has lifted from the bone near the fracture and a large number of mitotic figures is present in the deeper layer of periosteal fibroblasts. Silver impregnation shows a fine network of fibrils connecting the outer layer of the periosteum to the bone while the beginnings of bone matrix and cartilage formation are visible. At this stage the ground substance stains green-blue with Alcian blue and faintly with PAS. By 4 days the callus is increased in size, the periosteum is separated further from the bone and new bone is now forming in a wedge, the base of which is near and the apex away from the end of the fractured bone (Fig. 6). This new bone stains intensely positively with PAS and negatively with Alcian blue except at the margin where an osteoid border stains positively with Alcian blue and negatively with PAS. At high magnifications the PAS staining of new bone is shown to be due to the accumulation of fine granules of less than 1μ in size. These granules stain also with Ehrlich's haematoxylin. In some sections, at the edge of the new bone in the fracture area, cartilage which stains intensely with Alcian blue is forming. Joining the two ends of the fractured bone are fine agyrophilic fibres—except in the central area where removal of clot and debris continues by ingrowing granulation tissue. These collagen fibrils are laid down in association with plump fibroblasts containing PAS positive granules in their cytoplasm. The ends of the old bone are beginning to resorb ; there is increased vascularity and osteoclasts are seen at the bone edges.

At 6 days the callus is larger, new bone forming both on the periosteal and endosteal surfaces so that a collar of bone surrounds the ends of the fracture. This new bone is now thicker and newly formed bone trabeculae are orientated at an angle of the order of 60° to the old bone. Considerably more cartilage than usual has formed at the fracture site, particularly at the posterior aspect of the tibia. At about this time removal of this cartilage begins by the processes usually seen during intracartilagenous ossification of long bones. Owing to irregularity of arrangement of cartilage cells this process does not proceed in the same orderly manner as is seen at epiphyses during growth. The cartilage matrix usually becomes PAS positive at the edge where vessels are growing into it. This is due to the accumulation of PAS positive granules in the matrix.

All the processes described proceed until the twelfth day when the callus reaches its maximum size (Fig. 8). At this stage, in the rat, union is still by

connective tissue (Fig. 2), there being a greater amount in the anterior part of the fracture (which is the convexity of the angulation) than the posterior (which is the concavity) and it is orientated at right angles to the long axis of the bone. Resorption of the new bone is now taking place and a new phase of bone formation can be seen. This remodelling process consists of removal of new bone and its replacement, in part, by a matrix of more mature appearance; the ratio of matrix to cells is increased and it fails to stain with PAS or Alcian blue.

By 16 days there is considerably more mature bone particularly near the fracture where replacement of cartilage proceeds. The marrow is becoming less active and haemopoietic cells are being replaced by fat.

The picture seen at 20 days is that of a partly healed fracture where cartilagenous union has taken place in the posterior aspect (Fig. 12) but union is only by a thin band of dense connective tissue anteriorly. Removal and replacement of cartilage by bone, particularly on the posterior aspect of the callus, continues so that considerable remodelling has now taken place (Fig. 10).

Examination of the epiphysis of both the fractured (Fig. 13) and adjoining bones at the end of the experiments show that they are narrower than those in the normal animal.

Aminoacetonitrile Treated Fracture

By 4 days the periosteum adjacent to the fracture is greatly increased in thickness compared with that of the normal animal (Fig. 7). This is associated with rapid proliferation of periosteal cells and formation of considerable matrix containing finer fibrils than is normal. Bone matrix formation is considerably reduced in amount whilst, at the site of fracture, large numbers of connective tissue cells are forming cartilage. Here connective tissue fibrils are almost absent and the cells are arranged irregularly and vary greatly in size.

By 6 days numerous bone trabeculae have formed which, although arranged and orientated in the usual normal pattern, are considerably longer and thinner than in the control animal. PAS positive granules are now present in the matrix near the old bone and become progressively less toward the periosteum where new matrix, staining positive with Alcian blue, continues to form. The ends of the bone are connected by fine agyrophilic fibres except for the central area where debris is being replaced by granulation tissue. The callus continues to increase until by 12 days its diameter is about twice normal (Fig. 9). At this stage new bone is continuing to form at the periosteal margin; the bone matrix being laid down has thinner trabeculae than normal and numerous PAS positive granules, slightly bigger than those in the control animal, can be seen in the calcifying trabeculae. Extensive resorption of cartilage and new bone takes place although its replacement by a new layer of mature PAS negative matrix does not occur at the same rate as in the normal animal. The two ends of the fractured bone are widely separated by a layer of fine agyrophilic connective tissue fibrils in which only a few mature collagen bundles are seen (Fig. 3).

The epiphyseal cartilage is beginning to show the characteristic changes of the rat with lathyrous poisoning (Menzies and Mills, 1957).

At 14 days in the AAN-treated rat, resorption of the original bone forming the callus continues as shown by widely dilated vessels and the appearance of numerous osteoclasts. There is little replacement of this bone.

By 16 days large irregular haematoxylin and PAS postive granules are seen

at the margin of the new bone which is forming directly from fibrous connective tissue. They give the bone a distinctive granular appearance and when calcification has occurred some of them are associated with the canaliculi.

At the end of the experiment on the twentieth day the callus is extremely large and distorted. Most of the original bone and cartilage has been removed leaving only a thin shell of bone at the periphery of the callus and a few thin trabeculae in the main mass of the callus which is composed principally of active haemopoietic tissue (Fig. 11). New bone continues to form from connective tissue at the junction of bone and connective tissues. PAS positive granules are now 2–3 μ in diameter and in places are packed tightly together; their spherical outlines are sometimes distorted by pressure of adjoining globules (Fig. 20). They are still prominent in the fully formed immature bone giving it a granular appearance even when more homogeneous (lamellar) bone has been laid down around it (Fig. 19). This more mature bone contains no such granules. Sections stained to demonstrate bone canaliculi show that they are dilated and at junctions or where they divide large varicosities are present; however canaliculi are not seen in the area where the granules are beginning to form but only in areas where bone has presumably calcified. Here the globules are associated closely with the canaliculi. In some sections the space between the osteocyte and the matrix wall enclosing it is filled with PAS positive granules.

The ends of the bone are separated by a wide layer of connective tissue (Fig. 14) particularly in the anterior aspect of the callus. This connective tissue consists largely of agyrophilic fibres separated by wide spaces and occasional bundles of dense collagen are seen running through it (Fig. 5). Some areas stain homogeneously with PAS, collagen fibres are not stainable; even in silver stained sections they are not very obvious. The fibroblasts are now inactive and spindle-shaped though a few are still plump and contain PAS positive granules.

Most of the cartilage first formed has been replaced by bone. The epiphysis of both the fractured and control leg show gross changes characteristic of the action of AAN; irregular islands of distorted cartilage cells form the widened epiphyseal cartilage (Fig. 15).

The Withdrawal of AAN

In this group the AAN was administered for 12 days and then discontinued. Two days later (that is, on the fourteenth day) there are noticeable differences from the animals in Group 2. The removal of bone is less and more mature bone is being laid down. By 4 days (the sixteenth day of the experiment) the fractured ends are closer together particularly at the posterior aspect of the callus where remnants of clefts in the connective tissue are now seen between adjoining cartilage masses. The collagen on the anterior aspect is denser and stains less deeply with silver; bundles of mature collagen are running through the tissue.

After 6 days (the eighteenth day of the experiment) considerably more bone is formed, although it is still less than in the control; the bone fragments are closer together and the collagen joining them is denser. Much more cartilage has formed than in the continuously treated animal. By this time the epiphyseal cartilage has partially returned to normal. The islands of excess cartilage formed previously have been removed and replaced by a vascular connective tissue and the general pattern is now normal; although distorted metaphyseal bone trabeculae show the previous influence of AAN.

At the end of the experiment on the twentieth day, 8 days after the withdrawal of AAN, the callus is still considerably larger than normal. New bone is still being formed, particularly between the bone fragments at the junction of the connective tissue and bone. PAS positive globules, although still slightly larger than normal, are greatly reduced in size (Fig. 21). They appear as dense masses which, after their initial formation, gradually merge into the bone structure and are less obvious than those in the continuously treated animal. The bone canaliculi are now normal in arrangement, although the number of globules is too great to be accounted for easily by their attachment to canaliculi. The distance between the ends of the fractured bone on the anterior aspect is less than in the continuously treated animal (Fig. 16), whereas in the posterior aspect union is now progressing by cartilagenous union. The connective tissue is now matured and forms dense bundles of collagen fibres (Fig. 4) in contrast with the continuously treated animal where it is loosely arranged and immature (Fig. 5). Fibroblasts are spindle-shaped and contain few PAS positive granules.

The width of the epiphyseal cartilage has now returned to normal (Fig. 17).

DISCUSSION

Selye's (1957*b*) finding of giant formation in the AAN treated rat during the healing of fracture of the tibia is confirmed. There is, however, a considerable difference between tissue changes in the rat treated continuously and the one where, as was done in Selye's experiment, AAN is administered for 12 days and then withdrawn. This difference is sufficient to mask the primary site of the action of AAN in the animal in which administration ceases before the end of the experiment. In the animal treated with AAN the lesion occurs in connective tissues during the early stages of callus formation and is associated with a reduction in strength and increased mobility of the fractured leg. This is in agreement with the observations of Enzinger and Warner (1957) who showed that during healing the wounds in the lathyrous animal have a low tensile strength and those of Mielke *et al.* (1957) who found that croton oil pouches collapse in β aminopropionitrile (BAPN) treated rats due to abnormal formation of connective tissues. During callus formation this reduction in the strength of connective tissues is associated with continued mobility of the fractured leg leading to a constant stimulus for proliferation of fibroblasts and a delay in their metaplasia to osteoblasts and chondroblasts. One indication of this is the initial formation of long thin trabeculae of bone in the callus.

As the callus continues to enlarge all the stages seen in the normal rat are present, but grossly exaggerated. The initial bone formed in the callus is rapidly removed during the remodelling stage and very little mature bone replaces it. This results in the formation of a giant callus—a mass of tissue consisting of a peripheral thin shell of bone enclosing haemopoietic and fat tissue ; the two ends of the fractured bone are thus connected by mechanically inadequate connective tissue.

When AAN is withdrawn after 12 days' administration there is a reduction in mobility and slowing in the rate of formation of bulk of callus—even after 4 days the connective tissue is formed of denser bundles of recognizable collagen. This rapid return to normal strength and appearance has also been described in healing wounds when AAN was withdrawn (Enzinger and Warner, 1957).

In the callus following withdrawal of AAN a considerable amount of new bone forms round the original bone. At the same time new bone continues to form directly from connective tissue of the callus mass so that although the callus is larger than normal it is becoming well organized.

The epiphyseal cartilage also returns rapidly to normal and 6–8 days following the withdrawal of AAN the only sign of previous AAN administration is some irregularity in the metaphyseal bone.

It is not yet known whether the specific metabolic defect in lathyrous animals is in the collagen or the mucopolysaccharide component of connective tissues. Alterations in the appearance of ground substance have been demonstrated in previous experiments (Menzies and Mills, 1957 ; Hurley *et al.*, 1958), but there is no agreement as to whether this is an actual quantitative increase (Menzies and Mills, 1957 ; Churchill *et al.*, 1955 ; Dasler, 1957) or not (Ponseti *et al.*, 1956) ; nor is it clear from this study of callus formation. In the present study the finding of PAS positive globules of material is particularly obvious during the process of calcification in the callus. These have been described previously by Selye (1957*a* and *b*). In the present study these globules are obviously a bizarre form of the normal calcifying process where small granules are present at the transition zone between the fibrous matrix and calcifying bone. The presence of mucopolysaccharide at the site of incipient calcification and its different staining properties following calcification (Sylvèn, 1947) have been noted previously, although Rubin and Howard (1950) have pointed out that its disappearance following calcification is only due to a masking effect of calcification—de-calcification leads to the restoration of its staining properties. They suggested that this material is chondroitin sulphuric acid which helps to fix calcium prior to calcification. Attempts to stain both canaliculi and globules at the early stage of calcification reveal that where globules first appear no canaliculi are seen—but later in the process it is possible to stain parts of canaliculi and globules. This confirms Selye's observation that some globules are seen attached to canaliculi ; but others apparently are not and are most prominent in the developing osteoid tissue where it is not possible to stain canaliculi. It is possible that during the calcification process this mucopolysaccharide is produced by osteocytes or osteoblasts and proceeds either along canaliculi or out into surrounding intracellular spaces to play an important part in calcification. In the AAN-treated animal the production of mucopolysaccharide is abnormal and once produced globules can still be stained in woven bone, long after they can no longer be seen in the control animal.

When AAN is withdrawn a different distribution of these globules is seen. At the bone edge where new bone formation continues the globules are smaller and more deeply stained although still tightly packed together in the clumps covering the fibrous matrix. In bone formed earlier, when AAN was still being administered, these globules vary in size and do not completely cover the underlying matrix. Here they are attached to canaliculi and give appearances which resemble more closely the picture described by Selye (1957*c*).

In contrast with the bone formed from connective tissue during AAN treatment, that formed during the remodelling process appears normal although it is considerably reduced in amount. Canaliculi are normal and globules of PAS positive material absent. There are several possible explanations for this difference : that globules are less obvious in slowly forming bone, that calcification has not yet taken place or that the two processes of bone formation are different—bone

formed directly from connective tissue being more susceptible to the action of AAN by virtue of a change in ratios of fibrous matrix to bone mucopolysaccharide.

No finality has yet been reached on the question of whether collagen formation is altered in the lathyrous animal. Dasler (1957) found that hydroxyproline was normal in amount in the femur of the rat, whereas it was lowered in the wall of the pouch formed by injection of croton oil (Mielke *et al.*, 1957). Histological studies suggest that it is fragmented (Menzies and Mills, 1957) and that delay in maturation occurs (Hurley *et al.*, 1958). In this present study there is a delay in the appearance of mature collagen and bone, while bone formed directly from connective tissue is abnormal. In the early stages of callus formation there are alterations in the ratio of cells to fibres and ground substance, the number of cells being greatly increased. However it is not until the later stages that the ratio of mucopolysaccharide to fibres appears abnormal when homogeneous PAS positive material surrounds and masks the staining reactions of the immature collagen forming the connective tissue between the ends of the fractured bone. Similar changes have been noted previously during wound healing (Enzinger and Warner, 1957) in the formation of croton oil pouches (Mielke *et al.*, 1957) and in abscess walls (Hurley *et al.*, 1958).

The formation of giant callus during AAN administration reflects alterations in the fibrous matrix which, in the early stages, is reduced relative to the number of cells and during the late stages abnormal accumulations of PAS positive staining material are seen both in connective tissue and bone formed from connective tissues. Further decisions on the precise site of action of AAN will probably be dependent on combined histological and biochemical studies.

SUMMARY

Fracture of a bone in the leg of the rat (the tibia) heals in the same manner as in any incompletely immobilized bone, that is, there is good callus formation which shows considerable cartilagenous development.

During AAN administration there is excessive formation of poorly-formed fibrous tissue, cartilage and bone. This results in abnormalities of callus formation and produces a soft unstable tissue in which the mechanical stresses due to the use of the leg (supported mainly by the fibula) are much greater than usual. This is indicated by a deformity that occurs at the fracture site and results in the formation of a mass of tissue due to connective tissue proliferation stimulated by the mechanical stress. The final result is a giant callus consisting of a thin outer shell of bone held together at the fracture site by poorly-formed connective tissue.

As soon as AAN administration ceases, normal repair begins with the laying down of collagenous intercellular substance and the formation of bone. This is denser bone than is usual at this stage of the callus formation. This rebound phenomenon emphasizes the importance of observing all stages in the development of any phenomenon.

The mode of action of AAN is still uncertain, but observations to date suggest a disturbance in the formation of collagen and bone from the ground substance of developing connective tissue. This is particularly obvious in the forming bone where large globules of PAS positive material are present in the calcifying zone; these return to near normal size following the withdrawal of AAN.

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THE PROPERTIES OF CRYSTALLINE DIPHTHERIA TOXIN-PROTEIN: OBSERVATIONS ON THE PHOSPHATE-STABLE ANTIGEN

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POPE AND STEVENS (1958*a*) studied the effect of heat denaturation, chemical action, and enzyme action, on crystalline toxin-protein. By means of gel-diffusion tests they showed that this purified toxin contained several antigens. They also reported an apparent doubling of the Lf value when crystalline toxin-protein was submitted to the action of tribasic potassium phosphate. This work has been extended and the changes taking place have been measured by optical methods.

MATERIALS AND METHODS

Crystalline diphtheria toxin-protein

This was prepared as described by Pope and Stevens (1958*b*) and was a 2 × crystallized preparation.

Optical methods

The apparatus and methods described by Pope and Stevens (1953) were employed. The total volume of the toxin-antitoxin mixtures was reduced from 25 to 10 ml. to economize in material. Except where indicated the amount of antitoxin was constant at 200 flocculating units in the final volume of 10 ml.

Flocculation tests

In the optical method the amount of antitoxin is held constant and the amount of toxin varied for each mixture. This is the reverse of the procedure in the Ramon test. These two methods are known as "constant antibody (α procedure)" and "constant antigen (β procedure)" (Marrack, 1938). Timmerman (1934) showed that for diphtheria toxin the α and β methods gave values which differed by about 10 to 15 per cent. For consistency in this work the original crystalline toxin-protein and any modifications prepared from it were tested by both the α and β methods. In the preparation of toxin-antitoxin mixtures for optical examination only values obtained by the α procedure were used because in these mixtures the antitoxin was the constant component.

In vivo *tests*

The number of Lr doses per ml. was determined as described by Glenny (1931).

Antitoxin

All the antitoxin used was pepsin-refined (Pope, 1939).

Preparation of mixtures

Toxin and antitoxin solutions were accurately measured into a 10 ml. volumetric flask and made to volume with 0·9 per cent w/v sodium chloride. After mixing, the contents of the flask were transferred to small stoppered bottles. One 10 ml. volumetric flask was used

throughout the work to reduce errors. The mixtures were heated at 37°. After treatment with K_3PO_4 the toxin flocculates more slowly; care was taken to see that the reaction was complete before the final readings of optical density were made.

EXPERIMENTAL

The observations reported by Pope and Stevens (1958*a*) were based on the interaction of 5 per cent w/v K_3PO_4 and crystalline toxin-protein at room temperature and required 20–24 hr. for completion. It was later found that the reaction could be carried out at 35° and completed in 6 hr. This higher temperature was more convenient for experimental work since it allowed the examination of a series of samples over a 6 hr. period.

To a solution of crystalline diphtheria toxin-protein, containing 1900 flocculating units per ml. as determined by the α procedure, was added 5 per cent w/v K_3PO_4 and the mixture heated to 35°. Samples taken after varying intervals were adjusted by means of 7 N. phosphoric acid to pH 8 (glass electrode). These were then examined for their apparent Lf values by both the α and β procedures. A marked change in the apparent Lf value was found after 3 hr. at 35° which appeared to be complete by 6 hr. The values as determined by the α procedure were 0 hr.—1900, 3 hr.—3270 and 6 hr.—3410 units per ml.

In addition to the control untreated toxin, samples after 3, 4 and 6 hr. at 35° were selected for detailed examination by the determination of the optical density of a series of toxin-antitoxin mixtures, covering the range 20 to 300 per cent toxin to antitoxin. The results obtained are shown in Fig. 1 curves A, B, C and D. For curves A and B the antitoxin was held constant at 200 units per 10 ml. of final mixture. Owing to the reduced amount of floccule precipitate produced after 4 and 6 hr. treatment with 5 per cent K_3PO_4 the amount of antitoxin was increased for curves C and D to 400 units per 10 ml. of final mixture. To simplify the interpretation of changes produced by 5 per cent K_3PO_4 the 3, 4 and 6 hr. samples were considered as still having the same flocculation value, 1900 u./ml., as the original toxin, and all the mixtures for optical examination were prepared on this basis.

Inspection of Fig. 1, A shows that the curve for the interaction between the crystalline toxin-protein and the antitoxin was irregular. In this respect it agrees with the observation of Pope (1954). The first flocculating point in this and the other curves is indicated by the arrows. After 3 hr. at 35° with 5 per cent K_3PO_4 curve B shows that considerable change has taken place in the flocculation reaction with antitoxin. The point of first flocculation had changed as indicated by the arrow, and the amount of floccule precipitate was reduced. Since no further change in optical density occurred with time it was assumed that the reaction was complete. Curves C and D show the changes that were found in the toxin at 4 and 6 hr. at 35°. After 6 hr. treatment with 5 per cent K_3PO_4 at 35° curve D shows that the flocculation was now restricted to a symmetrical flocculation curve with its peak value at 60 per cent toxin to antitoxin, corresponding to the point of first flocculation. Some degree of opalescence was present in the toxin after 6 hr. treatment at 35°, and the blank value for the toxin alone, present in the mixtures, is shown by the dotted line in curve D.

From these curves it is clear that one component present in the original crystalline toxin-protein is stable to this treatment and retains most of its flocculating properties. By comparison with the curve for the original toxin it would appear that this antigen is responsible for the reaction in the " flocculation envelope "

in the region of one equivalent toxin to two of antitoxin (TA_2) and that it accounts completely for the apparent increase in Lf value reported by Pope and Stevens (1958*a*).

The properties of crystalline toxin-protein containing 5 per cent K_3PO_4 after 6 hr. at 35° were investigated more fully by determining the ability of this product

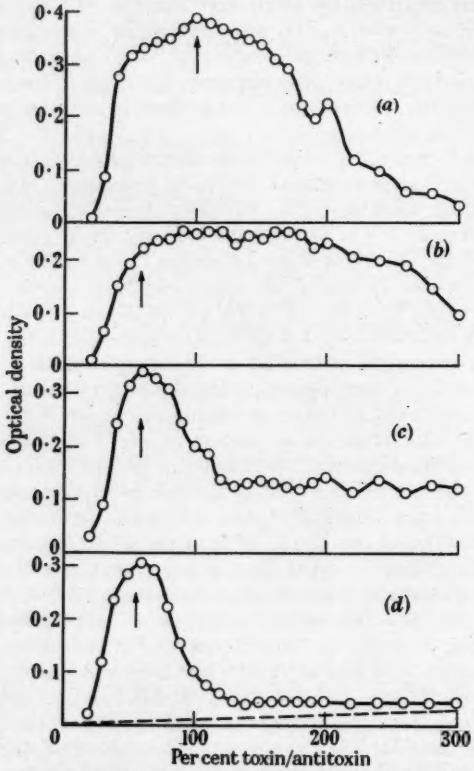

FIG. 1.—A. Control curve for crystalline toxin-protein and refined peptic-antitoxin. Antitoxin constant at 200 units/10 ml. in final mixtures. B. Crystalline toxin-protein with 5 per cent K_3PO_4 at 35° for 3 hr. Antitoxin constant at 200 units/10 ml. in final mixtures. C. As B after 4 hr. at 35°. Antitoxin constant at 400 units/10 ml. in final mixtures. D. After 6 hr. at 35°. Antitoxin constant at 400 units/10 ml. The dotted line shows the optical density for the control series containing toxin only; at 6 hr. some opacity was present in the phosphate treated toxin.

to combine with the specific antitoxin as determined by *in vivo* tests. Results, shown in the table, indicate that the 6 hr. sample still retains considerable combining property for the specific antitoxin, although tests had shown that the number of Lr doses per ml. had changed from an original value of about 2000 to less than 2 Lr doses per ml. as a result of phosphate treatment. The rapid loss of specific toxicity without a complete loss of combining power suggested that some form of toxoiding had taken place.

TABLE.—*The Combining Property with Antitoxin of* (a) *the Original Crystalline Toxin-protein and* (b) *the Same Containing* 5 *per cent* K_3PO_4 *after* 6 *hr. at* 35°

Antitoxin		Material added	Antitoxin in mixture (*in vivo* tests) (units/ml.)
200 units/ml. 1 volume	+	Saline 1 volume	105
Ditto	+	Original toxin 200 units/ml. 1 volume	4
,,	+	Phosphate-treated toxin 200 units/ml.* 1 volume	40

* This value is based on that of the original toxin and not on the increased apparent Lf value found after treatment. The phosphate-treated toxin contained less than 2 Lr doses per ml.

Two further experiments were carried out using the optical method in an attempt to gain more information about the antibody combining properties of the antigens modified by the phosphate treatment. In the first of these a range of mixtures was prepared in the following way. Volumes of the toxin (with 5 per cent K_3PO_4 and heated for 6 hr. at 35°) were measured to cover the range 20 to 300 per cent toxin to antitoxin. The treated toxin was assumed to have its original value of 1900 u./ml. The antitoxin (200 units constant) was then added and the mixture was allowed to react for 24 hr. Then a similar range of volumes of the original untreated toxin were added corresponding to the range 20 to 300 per cent toxin to antitoxin. Each mixture was then made to a volume of 10 ml. with saline. After completion of the reaction the optical density of each mixture was measured. Fig. 2 shows the result obtained. Floccule precipitate was present only in mixtures from 30 to 90 per cent toxin to antitoxin. This curve differs markedly from that of the control toxin, Fig. 1, A, and shows that apart from the area where the phosphate stable antigen is present, the part of the curve from 100 to 300 per cent toxin to antitoxin has been substantially altered.

The second experiment was made in the same way except that the range of control toxin was mixed with the range of phosphate treated toxin before the constant amount (200 units) of antitoxin was added and the volumes adjusted to 10 ml. In this way both the control toxin and the phosphate toxin were competing simultaneously for the antibodies. Fig. 3 shows that the presence of the phosphate treated toxin modified considerably the curve obtained, as can be seen by comparison with Fig. 1, curve A. Floccule precipitate was present in mixtures containing from 30 to 170 per cent toxin to antitoxin. Both these results indicate that apart from the phosphate stable antigen shown in Fig. 1, D the other antigens present have lost their ability to flocculate with their antibodies but not all their power to combine with them.

An attempt was made to isolate the antibody to the phosphate stable antigen in the following way. Toxin (5 per cent K_3PO_4 and 6 hr. at 35°) was mixed with antitoxin in proportions corresponding to the first flocculating mixture for this system (Fig. 1, D). After complete flocculation the mixture was centrifuged. The supernatant had lost 30 per cent of its flocculating value as determined by tests against toxin. In gel-diffusion tests it gave a negative reaction to the phosphated toxin used to prepare the floccules showing the complete removal of antibody to the phosphate stable antigen. The floccule precipitate was washed with saline

and resuspended in saline before treatment with pepsin at PH 3·0 (Pope and Healey, 1939) to liberate the antibodies. Using phosphate-toxin, tests showed that the recovered antibody solution contained 340 apparent flocculation units per ml.; *in vivo* tests showed the presence of 70 units per ml. of true antitoxin. This difference indicated an increased concentration of the antibody for the phos-

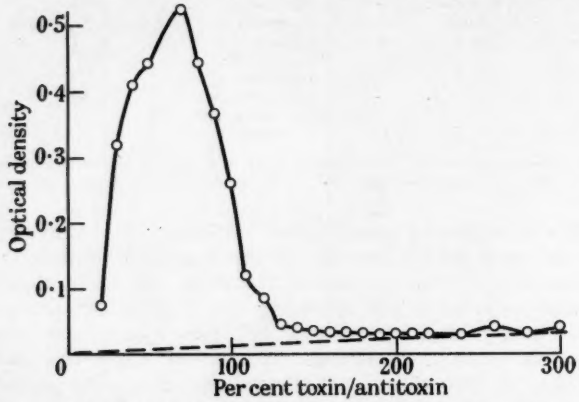

FIG. 2.—The range of phosphate toxin (6 hr. at 35°) was added to the antitoxin and 24 hr. allowed for combination before adding a similar range of the original toxin. Antitoxin constant at 200 units/10 ml. in the final mixtures. Dotted line shows density of the toxin controls.

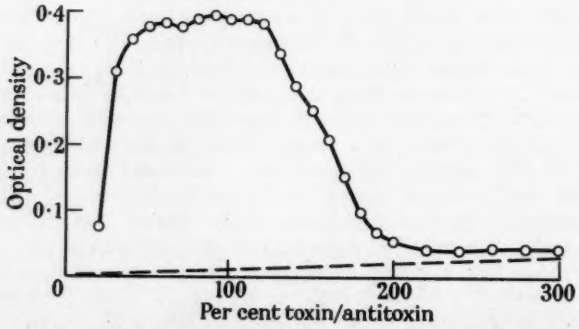

FIG. 3.—The range of phosphate toxin (6 hr. at 35°) was first mixed with a similar range of the original toxin before adding the antitoxin. Antitoxin constant at 200 units/10 ml. in final mixtures. Dotted line shows density of toxin controls.

phate stable antigen, together with some contamination with specific antitoxin. From the observations of Pope and Stevens (1953*a*) this result is not surprising. It has been shown here that although some antigens have lost their ability to flocculate following phosphate treatment their ability to combine with their antibodies has not been lost. The formation of non-flocculating polymers may be sufficient to ensure that some of these are incorporated into the gel-structure

produced by the phosphate-stable antigen and its antibody. Antibody giving a strong gel-diffusion line with phosphate toxin was present in this fraction.

DISCUSSION

Pope and Stevens (1953*a*) used the term " flocculation envelope " to describe the interaction of antigens and antibodies reacting to produce the floccule precipitation between diphtheria toxin and antitoxin. They showed (1953*b*) that after removal from antitoxic sera of the non-antitoxin antibodies it was possible to obtain non-flocculating antitoxin with *in vivo* properties. Based on these findings it was suggested that it might be possible to purify diphtheria toxin to a point where it would no longer flocculate with crude antitoxin. Although this has not yet been achieved there is evidence to suggest that crystalline toxin-protein (Pope and Stevens, 1953*c*, 1958*b*) contains a series of antigenically distinct components which are responsible for much of the floccule precipitate (Pope, 1957 ; Pope and Stevens, 1958*a*). Little is known about the properties of these antigens, but there is evidence (Pope, 1957) that the neutralization of the specific toxin by means of non-flocculating antitoxin does not affect the gel-diffusion line given by crystalline toxin-protein. More information regarding these antigens is desirable and in particular the part they play in the formation of the "flocculation envelope".

In this paper one of these antigens has been studied. It is stable to the action of K_3PO_4, or tetra sodium versenate (Pope and Stevens, 1958*a*) in 5 per cent concentration at 35° and shows a symmetrical flocculation curve with antitoxin. The survival of this antigen accounts for the apparent increase in the Lf value found when crystalline toxin-protein was acted upon by 5 per cent K_3PO_4 (Pope and Stevens, 1958*a*). In the titration of a fixed amount of antitoxic serum by additions of the crystalline toxin-protein, this antigen-antibody system reaches its equivalence point when about half an equivalent of the toxin has been added (TA_2) and this system is, therefore, responsible for flocculation on the excess antitoxin side of the " flocculation envelope ". It is not possible to state the contribution made by this system to the total flocculation ; the phosphate treatment has been shown to inhibit the flocculation of other antigen-antibody systems involved, and some reduction in flocculating ability may also be produced in this more stable system.

Other antigens, stable to trypsin (Pope, 1957), or pepsin (Pope and Stevens, 1958*a*), are known to be present in the crystalline toxin-protein and these have been studied by the method given here (Pope and Stevens—in preparation). Until all these antigen-antibody systems have been described it is not possible to discuss their contributions to the " flocculation envelope " or their relation to the specific toxin.

The antigens present in the crystalline toxin-protein should not be confused with the atoxic or P-proteins produced by growing *Corynebacterium diphtheriae* in the presence of excess iron in order to inhibit toxin production. During the purification of toxin (Pope and Stevens, 1958*b*) the P-proteins are eliminated. The antigens present in the crystalline toxin-protein are produced concurrently with the specific toxin.

SUMMARY

The action of 5 per cent K_3PO_4 on crystalline diphtheria toxin-protein has been studied using an optical method. It has been shown that the apparent increase of

Lf value is due to the presence of a phosphate stable antigen, which requires about 2 equivalents of antitoxin for optimal flocculation. It gives a symmetrical flocculation curve. The flocculating properties of other antigens are inhibited, but there is not a complete loss of their combining properties.

It was not found possible to isolate the antibody responsible for this system. Antibody recovered from the antigen-antibody floccules produced by this system was contaminated with specific antitoxin. Reasons for this are given.

After this paper had been prepared Poulik and Poulik (1958) reported their results using electrophoresis in starch-gel to separate the components present in diphtheria toxin. Their finding that the crystalline toxin-protein contained several distinct antigens is in agreement with those of Pope and Stevens (1958*a*) and the results reported here.

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HISTOCHEMISTRY OF A CONDITION RESEMBLING KWASHIORKOR PRODUCED IN RODENTS BY A LOW PROTEIN-HIGH CARBOHYDRATE DIET (CASSAVA)

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PROTEIN malnutrition, deficiency of protein with adequate carbohydrate, has been investigated at some length in both biochemical and morphological fields (Davies, 1952; Trowell, Davies and Dean, 1954). Little use, however, has been made of histochemical techniques in studies of these deficiency-states. Although crude by comparison with biochemical techniques the methods used in histochemistry are potentially valuable for the anatomical localisation of metabolic disorders. Furthermore no systematic investigation of the endocrine system appears to have been made in experimental protein deficiency.

The purpose of the present study was to investigate the extent of cytochemical changes in the liver and in certain exocrine and endocrine glands of rodents kept on a diet of cassava and to determine the effect of dietary supplements of certain proteins, aminoacids and vitamins in modifying the changes observed.

Cassava was fed to our animals since it is a low protein-high carbohydrate food and, furthermore, it is the staple food of large numbers of people in the tropics (Jelliffe, 1955). Moreover, it has been postulated that kwashiorkor is caused by such a low protein-high carbohydrate diet (Brock and Autret, 1952; Trowell, Davies and Dean, 1954).

METHODS

Diet

Two hundred adult male white mice, of weight 25–29 g., and 25 adult male white rats, of weight 200–220 g., were fed on cassava (gari, the dried grated fermented tuber of Manihot utilissima). This food contains approximately 1 per cent of protein and supplies 334 calories/100 g. (Data provided by the Western Region Federal Department of Commerce and Industry, Lagos, Nigeria.) Hot water was added to the cassava flour until a thick dough was formed: when cool this dough was fed to the animals. No restriction was placed on water intake or on the amount of food which each animal could eat. The rats were killed after 5, 7, 9, 11 and 15 weeks of the dietary regime: the mice were killed at the end of 4 weeks except for 10 animals killed at the end of the 2 weeks of the experiment.

Histochemical

Fixation

The animals were killed by decapitation and small pieces of the appropriate tissues were fixed immediately in neutral 4 per cent formaldehyde-saline. For the demonstration of protein, tissues were subject to 2–4 hr. fixation before clearing and embedding in paraffin.

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Techniques

For protein-bound aminoacids.—The *p*-dimethylaminobenzaldehyde-nitrite (DAB) method for trypotophan (Adams, 1957); Pollister's (1950) modification of the Millon method for tyrosine; Thomas' (1946) modification of the Sakaguchi method for arginine; the coupled alkaline tetrazonium method for tyrosine, tryptophan and histidine (Pearse, 1953); the performic acid-Alcian blue method for cystine and cysteine (Adams and Sloper, 1956) and the thioglycollate-ferric ferricyanide method for cystine and cysteine (Adams, 1956).

For carbohydrate.—The periodic acid-Schiff (PAS) method.

For enzymes.—Alkaline phosphatase (Gomori, 1952); acid phosphatase (Gomori, 1950) and non-specific esterase by the *α*-naphthyl acetate method (Pearse, 1953).

For nucleic acids.—Paraffin or frozen sections were stained for 20 min. in 0·06 per cent toluidine blue in 0·1 N acetate buffer at pH 4·0. The identity of ribonucleic acid (RNA) was confirmed by preliminary extraction with an 0·1 per cent solution of crystalline ribonuclease (Armour) in distilled water.

Duodenal Enzymes

After animals had been killed by decapitation, the pyloric sphincter was ligatured and a polythene cannula inserted at the duodeno-jejunal junction. One ml. of a 1 per cent solution of sodium carbonate was then injected through the cannula into the duodenum of a rat, or 0·5 ml. into that of a mouse. After the duodenal contents had been centrifuged the supernatant was tested for proteolytic activity by the gelatin film method of Gordon, Levin and Whitehead (1952). The activity of each sample tested was expressed as the reciprocal of the maximum dilution which caused complete digestion of the gelatin film.

Supplements to Diet

Groups of 5 mice each received supplements of the aminoacids; methionine, cystine, tryptophan, phenylalanine, valine and histidine. Each aminoacid was added to the diet at the rate of 1·25 g. per cent. Other groups were given supplements of vitamins (Hawkins

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EXPLANATION OF PLATE

FIG. 1.—Pancreas of rat after 15 weeks on cassava diet, DAB method for tryptophan, × 97. There is marked loss of zymogen-protein (compare with Fig. 1A).

FIG. 1A.—Pancreas of rat on stock diet, DAB method, × 97. Note the normal intensity of staining of the zymogen for tryptophan.

FIG. 2.—Stomach of rat after 15 weeks on cassava diet, DAB method, × 220. No protein can be seen in the peptic cells (compare with Fig. 2A).

FIG. 2A.—Stomach of rat on stock diet, DAB method, × 220. This shows intensely stained peptic zymogen granules.

FIG. 3.—Ileum of rat after 15 weeks on cassava diet, DAB method, × 220. No protein can be seen in the Paneth cells at the bottom of the crypts (compare with Fig. 3A).

FIG. 3A.—Ileum of rat on stock diet, DAB method, × 220. The zymogen of the Paneth cells is moderately well stained for tryptophan.

FIG. 4.—Submandibular gland of rat after 15 weeks on cassava diet, DAB method, × 97. Note the loss of zymogen protein from the markedly shrunk serous tubules (compare with Fig. 4A).

FIG. 4A.—Submandibular gland of rat on stock diet, DAB method, × 97. Note the intense staining of zymogen in the epithelium of the serous tubules.

FIG. 5.—Liver of rat after 7 weeks of cassava diet, Sudan IV, × 72. Note slight periportal fatty change.

FIG. 6.—Liver of rat after 9 weeks of cassava diet, Sudan IV, × 72. Note severe fatty change.

FIG. 7.—Liver of mouse after 4 weeks of cassava diet, DAB method, × 92. Note loss of protein from parenchymal cells (compare with Fig. 7A).

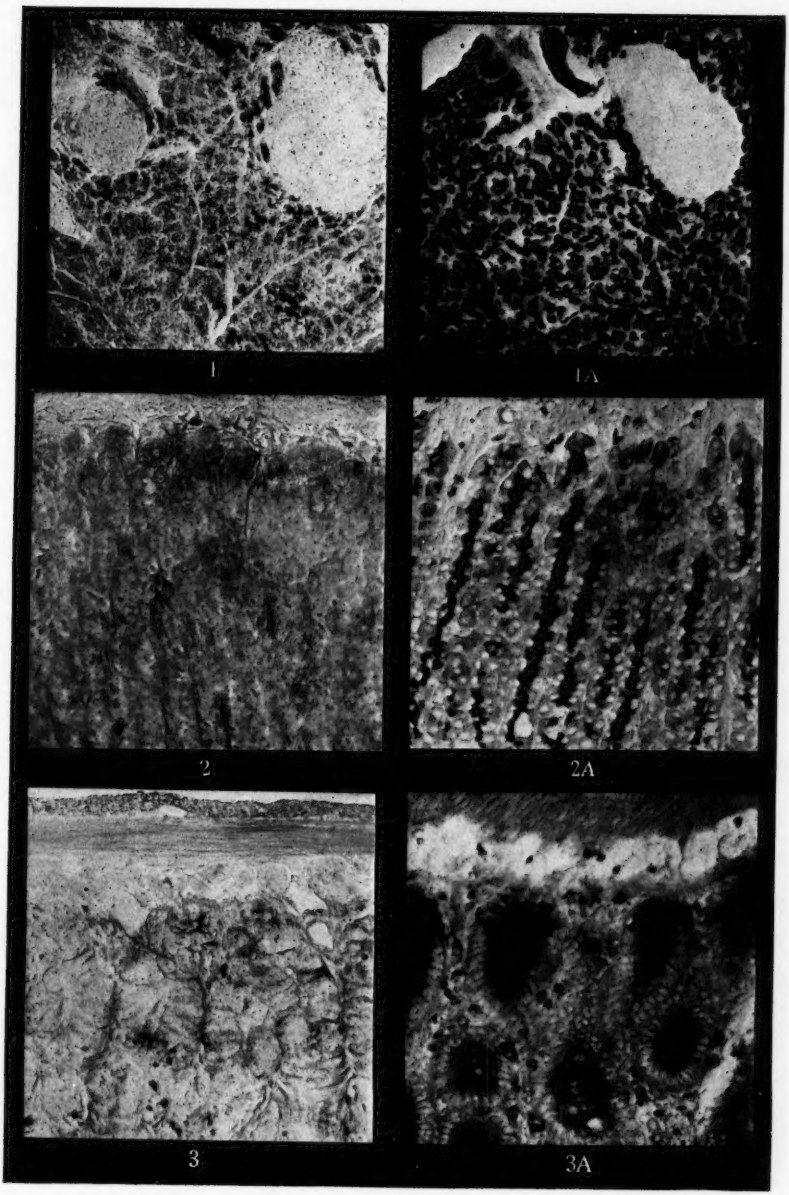
FIG. 7A.—Liver of mouse on stock diet, DAB method, × 92.

FIG. 8.—Testis of rat after 15 weeks on cassava diet, H. and E., × 257. Note the marked shrinkage of the interstitial cells: the seminiferous tubules appear normal (compare with Fig. 8A).

FIG. 8A.—Testis of rat on stock diet. H. and E., × 257.

FIG. 9.—Quadriceps muscle of mouse after 4 weeks on cassava diet, DAB method, × 106. There is no reduction in the intensity of staining of the fibres (compare with Fig. 9A).

FIG. 9A.—Quadriceps muscle of mouse on stock diet, DAB method, × 106.



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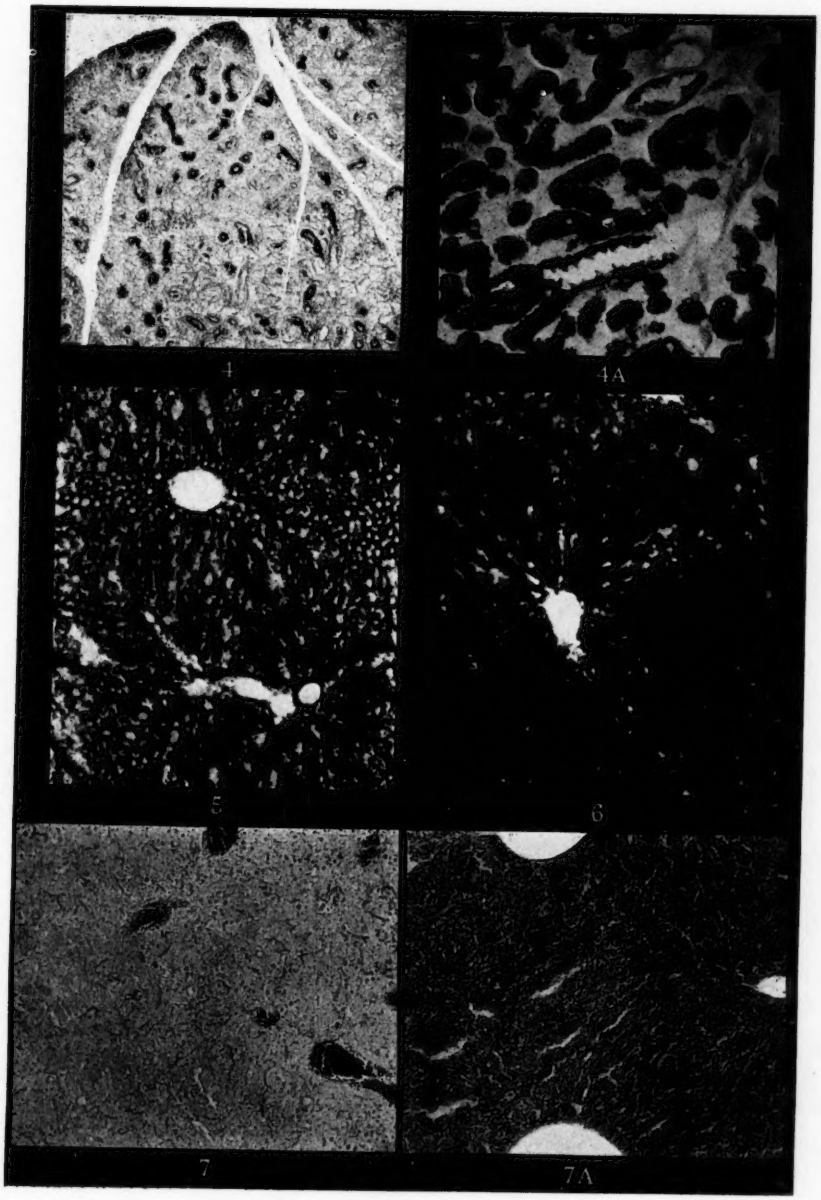

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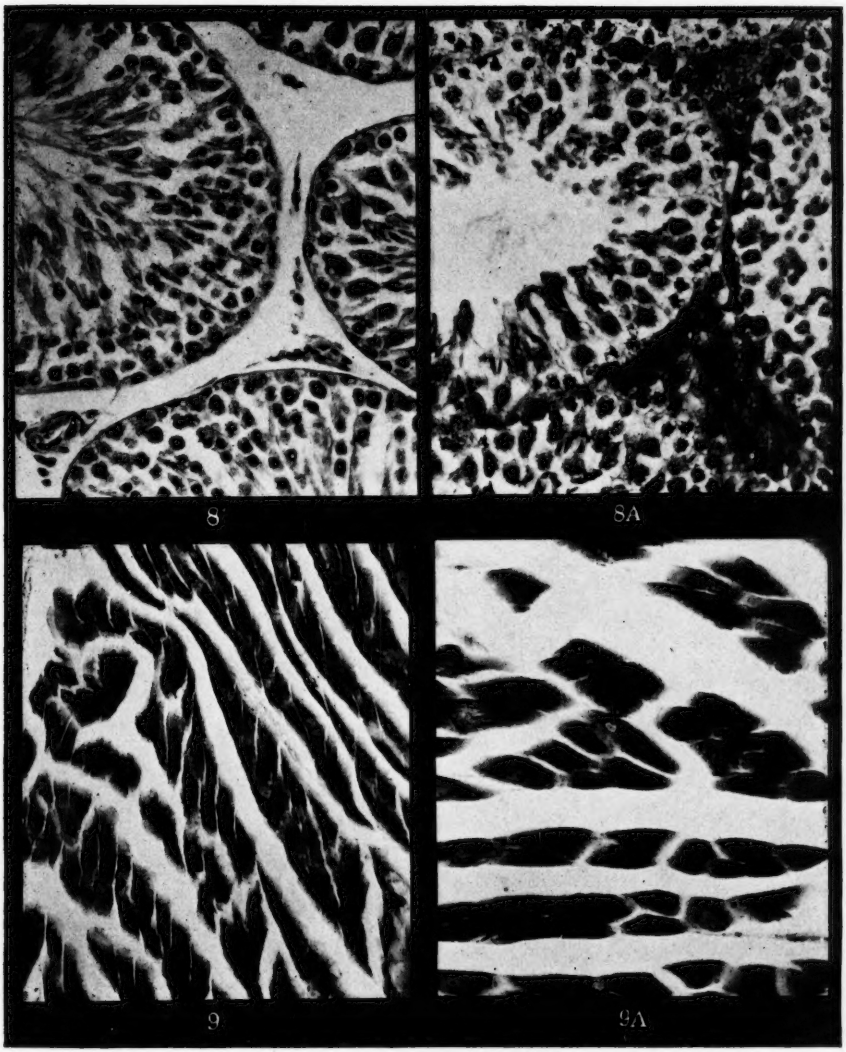

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mixture [1952] including choline and cyanocobalamin), aminoacids plus vitamins, dried milk (30 g. per cent), vitamin-free casein (15 g. per cent), gelatin (15 g. per cent), gelatin (15 g. per cent) plus aminoacids and vitamin-free casein (15 g. per cent) with added vitamin mixture.

Biochemical

Protein-nitrogen in liver and muscle was estimated by the micro-Kjeldahl method.

RESULTS

Exocrine glands

Pancreas.—In rats killed after 5 weeks on the diet, the pancreatic acini showed definite histochemical and morphological differences from control animals. Thus there was marked loss of stainable protein in the zymogen granules as shown by the DAB method (tryptophan) and some decreased eosinophilia. However, no loss of RNA was seen in sections stained by either buffered toluidine blue or haematoxylin and eosin.

In rats killed after 7 weeks on the diet some proliferation and widening of the ducts was seen in 2 out of 5 pancreases examined. This was associated with peri-acinar round-cell infiltration but not with fibrosis. However, closer examination of the areas of duct proliferation revealed that at least half of the apparently newly-formed ducts might be grossly shrunken acini. The appearance of the nuclei, the absence of mitoses and the presence of RNA in these acini (RNA is not present in the epithelium of normal ducts) supported this view.

The de-granulation of zymogen and the atrophy of acini (Fig. 1) grew progressively worse during the course of the experiment and there were, correspondingly, more areas of duct proliferation.

The changes observed in the pancreas of the mouse, on the diet for 4 weeks, were similar to those in the rat at 15 weeks. There was no loss of non-specific esterase activity from even severely shrunken acinar cells in the mouse.

Gastro-intestinal tract.—The morphological and histochemical appearances of the peptic cells of the stomach of rats, killed after 7 weeks on the diet, were in many ways similar to those of the pancreatic acinar cells. Thus there was a similar decrease in the size of zymogen granules and loss of protein (DAB method). As with the pancreas these changes increased progressively until the end of the experiment at 15 weeks (Fig. 2). No decrease was seen at any time in the intensity of staining of the basal chromidial substance (RNA) of these cells, although it was possible, as a result of cell-shrinkage, that the total amount of RNA in each cell was in fact reduced. Similar changes were seen in mice on diet for 4 weeks.

The changes in the Paneth cells of the ileum in both rats (Fig. 3) and mice followed a similar pattern to those in the pancreatic acinar cells and gastric peptic cells. Likewise no loss of RNA was detected in these cells. Loss of protein (DAB method) could be demonstrated in the Paneth cells of rats which had been 5 weeks on the diet. In these cells reduction in the intensity of staining of the granules with both PAS and eosin was only observed after the animals had been 9 weeks on the diet.

In PAS-stained sections of the ileum of rats which had been 7 weeks on the diet there was a reduction in the number of goblet cells as compared with the normal.

Salivary glands.—In the submandibular gland of cassava-fed rats and mice, loss of protein and eosinophilia from the zymogen granules followed a similar

course to that of the other secretory cells examined. Since the zymogen granules of " serous tubular " cells contained more protein than those of serous cells this change was more prominent in the tubular zymogen (Fig. 4). There was no corresponding loss of RNA from the basal zone of the serous cells. The mucous cells showed no morphological alteration or reduction in staining by PAS. The non-specific esterase activity in both serous and " tubular serous " cells was slightly reduced in mice kept 4 weeks on the diet, but there was no reduction in either acid or alkaline phosphatase activity.

Liver

Slight periportal fatty changes were seen in mice which had been kept 2 or 4 weeks on the diet. In the cassava-fed rat moderate (Fig. 5) to severe (Fig. 6) accumulations of periportal fat were seen from 7 weeks onwards. The amount of fat found bore no relation to the degree of malnutrition (Table I).

In the mouse liver a marked loss of cytoplasmic protein (DAB method; Fig. 7), RNA and non-specific esterase activity was noted after the animals had been 4 weeks on diet.

In the rat, however, it was not often possible to determine the extent of these changes since the parenchymal cells were usually so full of fat as to compress the surviving cytoplasm into a thin rim at the edge of each cell. In the few livers of this species where there was little fat, both protein and RNA were reduced as early as 7 weeks on the diet. Nitrogen estimations were therefore made on representative samples of the livers of rats in order to overcome the difficulties experienced in estimating protein histochemically. A loss of 35–40 per cent nitrogen occurred in the livers of rats which had been fed cassava for 7, 9 and 15 weeks (Table I). It was noted that the loss in protein nitrogen was in no way related to the severity of fatty infiltration. In contrast to the loss of non-specific esterase activity there was a marked increase in the staining of the liver with both acid and alkaline phosphatase methods. The accumulation of these phosphatases was mainly in the sinusoids and to some extent in nuclei. Neither the cytoplasm of the parenchymal cells nor the biliary canaliculi were stained appreciably by either method.

Endocrine glands

In mice kept on a diet of cassava for 4 weeks the proportion of basophil cells to acidophil cells in the adenohypophysis appeared normal The size and number of granules in each basophil cell were within normal limits. Likewise there was no alteration in the staining of the basophil granules by both the PAS-orange G and esterase methods. Accurate cell counts were not made and these observations rest upon the examination of 4 middle sections from the pituitaries of 5 animals.

The thyroid vesicles of mice kept on the diet for 4 weeks had a somewhat flatter epithelium than those of control animals. Where the epithelium was flattened non-specific esterase, acid and alkaline phosphatase activities were all reduced.

There was no reduction in the width of the adrenal cortex or of the proportion of zona fasciculata to reticulata in mice after 4 weeks on the diet. The amount of birefringent lipid in the cortex of cassava-fed animals appeared normal. Although no change was seen in the morphological appearance of cells in any of the indi-

vidual layers, there was a moderate reduction of the alkaline phosphatase activivity in the whole cortex. Acid phosphatase activity remained unaltered.

Hypertrophy of the pancreatic islets was not seen in either mice kept on a diet of cassava for 4 weeks or rats kept on the diet for 5, 7, 9, 11 and 15 weeks. The relative proportion of beta to alpha cells was not increased (aldehyde fuchsin). Acid phosphatase activity was so much increased in the islets of mice, which had been on the diet for 4 weeks, as to prevent the identification of either sinusoids or cells.

The cytoplasm of the interstitial cells (Leydig) of the testis was markedly shrunken (Fig. 8), poorly stained by the DAB method, and had lost most of its eosinophilia. These changes were seen both in mice which had been 4 weeks on the diet and in rats after 15 weeks of diet. The surviving cytoplasm, however, was stained rather more strongly by PAS than the normal. Both non-specific esterase and acid phosphatase activities were reduced in proportion to the loss of cytoplasm from the cell.

Other tissues and organs

No histochemical or morphological changes were seen in the seminiferous tubules of the testis in the mouse after 4 weeks of the diet and in the rat after 15 weeks (Fig. 8). In the tubules of animals, fed on the stock diet, a strong reaction for alkaline phosphatase was found in the nuclei of spermatogonia and in the region of the basement membrane; no difference was found in animals fed on cassava.

Although visceral and skeletal muscle appeared shrunken to the naked eye in mice after 4 weeks and in rats after 15 weeks of the diet, yet individual muscle-fibres were stained for protein (DAB method) as strongly as in control animals (Fig. 9). This was confirmed by the nitrogen estimations of muscle (Table I) for there was no apparent loss of protein (per unit weight) from the quadriceps muscles of cassava-fed rats when compared with the normal.

TABLE I.—*The Effect of the Cassava Diet on Liver and Muscle Protein and on the Extent of Fatty Change in Rats*

Weeks on diet	Number of animals	Liver protein mg. N/100 mg. wet weight	Liver fat (Sudan IV)	Number of animals	Muscle protein mg. N/100 mg. wet weight
7	2	2·15	++++	—	—
		2·19	++		
11	2	2·26	+	—	—
		2·38	+++		
15	4	2·20	++++	5	3·11
		2·37	+		3·28
		2·40	++++		3·33
		2·42	++		3·34
					3·52
Mean of all animals on cassava diet	8	2·30±0·11	—	5	3·32±0·13
Stock diet	5	3·45	—	5	3·16
		3·47			3·21
		3·50			3·27
		3·89			3·39
		3·91			3·44
Mean of animals on stock diet	5	3·64±0·21	—	5	3·30±0·11

The staining of epidermal and hair keratin for the individual aminoacids cystine, tyrosine and arginine was also not reduced in cassava-fed animals.

Duodenal enzymes.—The proteolytic activity of the duodenal juice (Table II) was reduced about 75 per cent in mice which had been on the cassava diet for 3 weeks. There was a similar reduction in rats which had been on the diet for 15 weeks.

TABLE II.—*The Effect of the Cassava Diet on the Proteolytic Activity in the Duodenal Juice of Rats and Mice*

(Expressed as reciprocal of maximum dilution required to digest gelatin plate)

Rats			Mice		
Normals	After 15 weeks of cassava diet	t and p	Normals	After 21 days on diet	t and p
155±28 (7)	44±9 (12)	$t=4\cdot6$ $p<0\cdot001$	60±4 (8)	15±5 (7)	$t=7\cdot3$ $p<0\cdot001$

Figures in parenthesis are number of animals used.

Effect of supplements (mice only)

The supplements added to the diet of cassava are presented in Table III. It will be noted that certain dietary supplements were added at the 14th day when the mice had lost 25–30 per cent of their initial weight, others were added from the beginning. The criteria which were applied to assess the beneficial effect of supplements were: rapid gain in weight (or, when the supplement was added at the beginning, no loss of weight); re-granulation of pancreatic acini (no de-granulation); re-granulation of the serous tubules of the submandibular gland (no de-granulation); increase in the cytoplasmic protein of the liver (no loss of protein).

Animals which satisfied all 4 criteria could not be distinguished, in regard to their general appearance and behaviour, from those on the stock diet.

No beneficial effect was obtained with supplements of aminoacids (individually or in combination), incomplete protein (gelatin) or a composite vitamin mixture. The supplement of gelatin, cystine, methionine, tryptophan and valine (*i.e.*, gelatin plus its deficient aminoacids) gave some degree of protection. In contrast, dried skimmed-milk or vitamin-free casein brought about nearly complete recovery.

DISCUSSION

Friedman and Friedman (1946) and Véghelyi, Kemény, Pozsonyi and Sós (1950) have reported that de-granulation and atrophy of the acinar cells of the pancreas occurs if rats are kept on a diet deficient in protein. In the present work a diet of cassava produced similar effects. The loss of tryptophan-rich protein from the granules of the pancreatic acini found in our experiments is probably related to the diminished proteolytic activity of the duodenal juice, for endopeptidases (trypsin and pepsin) are known to contain an unusually high proportion of tryptophan (Block and Bolling, 1945). Another factor which may also contribute to this diminution is the atrophy of Paneth cells, for it is thought that these exocrine cells secrete carboxypeptidases (Blaschko and Sanders, 1951).

TABLE III.—*The Effect of Supplements to the Cassava Diet in Mice*

Cassava plus :	Number of animals	Weight at end per cent.	Zymogen in pancreas and serous tubules	Protein in liver
No supplement :				
Killed at 14th day	10	−27	RR	RR
Killed at 28th day	20	−42		
Supplements from 14th to 28th day :				
Methionine (1)	5	−47	RR	RR
Cystine (2)	3	−39·5		
Tryptophan (3)	5	−43·5		
Valine (4)	4	−42		
Phenylalanine (5)	5	−44		
Histidine (6)	3	−44·5		
(1)+(2)+(3)+(4)+(5)+(6)	5	−39		
Dried skimmed milk	5	−7	N	N
Vitamin-free casein (7)	5	−1		
Vitamin mixture (8)	4	−46	RR	RR
(7)+(8)	5	−5	N	N
Gelatin (9)	4	−35·5	RR	RR
Supplements from 1st to 28th day :				
(8)	4	−38·5	RR	RR
(9)	5	−39		
(9)+(1)	5	−37·5		
(9)+(2)	4	−47·5		
(9)+(3)	4	−39		
(9)+(4)	5*	—	—	—
(9)+(1)+(2)+(3)+(4)	5	−26	R	R

Key : R = moderate loss ; RR = severe loss ; N = normal or nearly normal.

* All the animals in this group died on the 15th day (weight = − 34 per cent).

The submandibular gland was examined in preference to the other salivary glands, since this compound gland is known to contain, in both the rat and the mouse, unusual secretory intralobular ducts (the " serous tubules " of Grad and Leblond, 1949). The normal gland, in both species, is divisible into a lobe of PAS-positive apparently " mucous " acini ; a lobe composed of weakly PAS-positive serous acini and a large lobe, " mucous " in appearance, but presenting a well-developed duct system, whose epithelium consists of secretory cells containing large phloxinophilic PAS-positive granules. In the normal gland the granules of the serous tubules are intensely stained by the DAB method (tryptophan), while the granules of the serous cells are only moderately stained and those of the mucous cells are unstained by this method. Basal chromidial substance (RNA) is known to be a characteristic of serous cells only and RNA cannot be demonstrated in the mucous and serous tubular cells (Grad and Leblond, 1949).

Sreebny, Meyer, Bachem and Weinmann (1957) have shown that the secretion of the submandibular gland in the rat has proteolytic activity. We have found in both the rat and mouse, that digestion of gelatin occurs in the region of serous tubules when frozen sections of rapidly fixed submandibular gland are incubated on exposed blackened photographic plate in a water-saturated atmosphere. This suggests that the zymogen granules of the serous tubules are a source of proteolytic enzyme. It follows that the histochemical changes found in the submandibular glands of rodents kept on a diet of cassava may be associated with loss of proteolytic activity of the gland.

In preliminary studies with hormone supplements it appeared that the degranulation and atrophy of the submandibular serous tubules was related to the involution of the Leydig cells for when testosterone was administered to the protein-malnourished animal the serous tubules were histologically almost normal. This is in accord with the observations that castration of the male mouse (Lacassagne, 1940) and male rat (Grad and Leblond, 1949) leads to de-granulation and atrophy of these serous tubules, a condition which was corrected by testosterone. To what extent the atrophy of the Leydig cells in protein-malnutrition can be correlated with pituitary dysfunction is a subject for future investigation.

It was surprising that in the course of protein-malnutrition there was no loss of RNA from the zymogenic cells of the exocrine glands in question, for it is widely accepted (Davidson, 1953) that RNA is concerned with protein metabolism possibly as a carrier for the transfer of aminoacids (Shigeura and Chargaff, 1957). Non-specific esterase activity was also within normal limits in these exocrine glands. The exact physiological function of these esterases is not known, but Pearse (1956) has suggested that some of them may be intracellular peptidases. It would appear that in conditions of protein starvation RNA and some enzyme activity is maintained in the exocrine glands in order, possibly, that the organism may still be able to deal with any protein which becomes available to it.

While the thyroid and adrenal glands of mice on the diet both showed slight impairment of enzyme activity there was a considerable increase of one enzyme, acid phosphatase, in the pancreatic islets. It is possible that this may have been related to the large amount of carbohydrate eaten by these animals.

Fatty liver is known to occur in rodents kept on a protein deficient diet (Heller and Blackmore, 1952; Best, Hartroft and Lucas, 1955). The degree of fatty change is related to the time the animals are kept on the diet (Heller and Blackmore, 1952) but the time required for it to occur varies from species to species. Hence it is unwise to compare the degree of fatty change found in different species. Our results, however, confirm those of Shils, Friedland and Stewart (1954) that periportal fatty changes occur in rats kept on a diet of cassava.

The increase of alkaline phosphatase in the venous sinuses of the liver during protein malnutrition in the rat has been previously described by Ramalingaswami, Sriramachari and Patwardhan (1954). It is possible that this is attributable to failure of the liver to remove and excrete circulating phosphatase in the blood (Sherlock and Walshe, 1947).

Relationship of present results to previous findings in aminoacid and protein deficiency states.

The histological effects of a diet of cassava fed to rodents differed from that produced by a diet of maize meal and sour milk (Gilbert and Gillman, 1944). Animals fed on the latter diet developed marked testicular changes: the testis showed tubular degeneration and giant cells were seen. These changes are reminiscent of those found in tryptophan deficiency (Adamstone and Spector, 1950). It must be pointed out, however, that Gilbert and Gillman's animals were younger than ours.

It is known that cassava is deficient in certain aminoacids notably methionine and cystine (Bigwood and Adriaens, 1952). However, the addition of these supplements to the diet of mice which had been fed cassava for 14 days failed to have any beneficial effect. Supplements of the other aminoacids particularly deficient

in cassava (tryptophan, phenyl-alanine, histidine and valine) also failed to have effect. Moreover the lesions seen were unlike those described singly or collectively in the rat deficient in phenylalanine (Maun, Cahill and Davis, 1945*a* ; Schwartz, Scott and Ferguson, 1951), threonine (Scott and Schwartz, 1953), histidine (Scott, 1954) or leucine (Maun, Cahill and Davis, 1945*b*).

Only the addition of complete protein, *i.e.*, containing all the essential amino acids in proper proportion, was effective in reversing and preventing the histological changes induced by cassava.

Our results indicate that the effects produced by feeding an animal on a predominantly carbohydrate diet are due to deficiency of protein as a whole rather than to any individual component of protein. It is significant that the organs which were most affected by the cassava diet were those, such as the pancreas and liver, with the most rapid turnover of protein. In this connection it has been shown by Shemin and Rittenberg (1944) and by Sprinson and Rittenberg (1949) that the half-life of labelled liver proteins is about 7–10 days in the human and about 6 days in the rat. In muscle and skin, where we found no loss of protein in cassava-fed animals, this period is about 160 days in the human and 21 days in the rat. These authors concluded that, if the serum proteins are included, nearly half of the active turnover of protein in the organism is conducted by the liver. Likewise Tarver and Schmidt (1942), working on the dog, found that the amount of labelled methionine which was incorporated within unit time into protein of the intestinal mucosa, pancreas, liver and muscle was in the ratio 81 : 47 : 41 : 1 respectively ; it may be inferred that these figures reflect the rate of protein-turnover in these organs. Accordingly, the observations of Tarver and Schmidt and of Rittenberg and his colleagues would account for the marked loss of protein from the liver and pancreas of the malnourished animals in contrast to the sparing of those of muscle and skin.

Relationship to kwashiorkor

Doubt has been expressed by Davies (1952) concerning the relationship of protein malnutrition in the experimental animal to kwashiorkor in the human. There is, however, considerable biochemical evidence to show that these two conditions are closely related (Blackmore and Schnieden, 1957 ; Schnieden, Hendrickse and Haigh, 1958). Moreover the histological changes which have been described in kwashiorkor (Gillman and Gillman, 1954*a* ; Waterlow, 1948 ; Davies, 1948 ; 1952 ; Sriramachari and Ramalingaswami, 1953) bear some resemblance to those previously observed in experimental protein deficiency (Friedman and Friedman, 1946 ; Véghelyi *et al.*, 1950 ; Best *et al.*, 1955).

In our experiments we have been able to produce similar morphological changes to those described by Davies (1948, 1952) in human kwashiorkor, namely atrophy of the pancreas, salivary glands, small intestine, peptic and Paneth cells. Furthermore in the present experiments the proteolytic activity of the duodenal juice was diminished in animals kept on a diet of cassava. Similar findings have been reported in human kwashiorkor by Thompson (1952), Thompson and Trowell (1952) and by Badr El-Din and Aboul Wafa (1957). There is also a therapeutic similarity between the two conditions. Vitamin mixtures and methionine have been shown to be without effect in kwashiorkor (Gillman and Gillman, 1945*b*); Trowell, 1946 ; Waterlow, 1948) and treatment with complete protein essential

(Waterlow, 1948; Brock, Hansen, Howe, Pretorius, Davel and Hendrickse, 1955). Similar findings have been recorded by us in animals kept on a diet of cassava.

Nevertheless it might be argued that the changes found in rodents kept on a diet of cassava differ from those of kwashiorkor in that it has been suggested that the pancreas is affected before the liver in the human disease (Davies, 1948; 1952). This author found that pancreatic atrophy occurred before the onset of fatty liver. In contrast, in the present experiments, it has been shown that protein is lost from both pancreas and liver early in the course of protein-deprivation. This suggests that both organs are affected simultaneously. Thus, apart from species differences, it is likely that experimental protein deficiency and kwashiorkor are essentially similar.

SUMMARY

The effects on rodents of a diet of cassava have been investigated using histochemical and biochemical techniques.

There was loss of stainable protein from the zymogen granules of the pancreatic acini, the peptic cells of the stomach, the Paneth cells of the ileum, the serous tubular cells of the submandibular gland, from the parenchymal cells of the liver and from the interstitial cells of the testis of animals kept on such a diet. All these cells were atrophied and showed diminished eosinophilia.

No loss of RNA and no change in non-specific esterase activity was found in those exocrine glands in which these tests were done but in certain endocrine glands (insterstitial cells of the testis and thyroid) non-specific esterase activity was diminished. In the liver RNA and non-specific esterase activity were both reduced.

While alkaline phosphatase activity was moderately reduced in the adrenal cortex, there was a substantial increase of acid phosphatase activity in the pancreatic islets. In the liver both acid and alkaline phosphatase activities were increased.

The de-granulation and atrophy of the serous tubules of the submandibular gland was probably related to the involution of the interstitial cells of the testis for the serous tubules appeared nearly normal when testosterone was added to the diet.

The histological changes found in animals kept on a diet of cassava could not be corrected by supplements of incomplete protein, individual aminoacids, vitamins and lipotrophic factors. Only complete protein was effective.

The relationship of the syndrome, seen in cassava-fed rodents, to kwashiorkor has been discussed. The organs which are predominantly affected in both conditions are those with a rapid turnover in protein. Apart from species differences it is concluded that the two conditions are essentially similar.

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THE RELATION OF THE FOETAL KIDNEY TO THE FALL IN BLOOD PRESSURE OF HYPERTENSIVE RATS DURING PREGNANCY

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It has been established that during pregnancy the blood pressure of renal hypertensive rats falls substantially (Harrison, Grollman and Williams, 1940; Wilson and Byrom, 1941; Page, Ogden and Patton, 1941; Foa, Foa and Peet, 1942; Page, 1946). Depending on the litter size (Grollman, 1947) the blood pressure may be reduced to normal or even subnormal levels, and, if it is high at conception, a fall of 100 mm. Hg or more may occur. Any theory explaining renal hypertension must also account for such a striking reversal of the hypertensive process. At present many believe that two phases of experimental renal hypertension occur: an initial hypertension due to the renin-hypertensin system and a later hypertension resulting from the operation of an extra-renal mechanism. The fall in blood pressure in the pregnant rat occurs, however, whether the hypertension is of short or long duration.

Two groups of workers (Goldblatt, Kahn and Hanzal, 1939; Harrison, Grollman and Williams, 1940) from slightly different viewpoints ascribe the fall in maternal blood pressure to the antipressor activity of the developing foetal kidneys. Page (1947), however, showed that if the foetuses were surgically destroyed without damage to the placenta, hypertensive rats still showed a fall in blood pressure while the placentas were retained. There is much evidence, summarised by Page (1947), demonstrating that such animals with retained placentas are physiologically pregnant until the placentas are delivered at or near term. As a control for the changes in blood pressure caused by the operative removal of the foetuses (foetal ablation), hysterectomies and sham operations were carried out on groups of hypertensive rats. Grollman (1947) considered these controls inadequate, for he states that " the effect of operative shock involved in removing the embryos might account for the observed drop in pressure ".

The primary purpose of the present investigation was to re-examine the described effects of foetal ablation with more adequate controls for the consequences of operative trauma. It was found that the foetus was not responsible for the maternal fall in blood pressure. This result gave an opportunity to test a corollary of some recent findings (Mackaness and Dodson, 1957) that during the second half of pregnancy rats become progressively more refractory to the pressor action of renin. If the fall in blood pressure in the pregnant hypertensive rat and the refractoriness of the normal pregnant rat to renin are related, animals which have undergone foetal ablation should also give much reduced pressor responses to renin. This question has also been examined.

METHODS

Rats.—Albino rats bred in this laboratory from stock of Wistar origin were used.

Blood pressure was recorded in the unanaesthetised rats with a modification of Byrom's oscilloscope and the methods previously described (Dodson and Mackaness, 1957).

Hypertension was induced by placing a silver clip on the left renal artery in the manner described by Wilson and Byrom (1939). The gap between the limbs of the clip was 0·011 in. The right kidney was left intact.

Foetal ablation.—Under ether anaesthesia a small hole was made with a surgical needle into each amniotic sac and the foetus expressed through the hole.

Total ablation.—After removing the foetus as above, the placenta was removed through the same hole in the uterus.

Renin was prepared by Schales's (1942) method and given by intraperitoneal injection. By this route the pressor response is protracted and the peak response is reached about 20 to 40 min. after injection.

Details of the response to the intraperitoneal injection of renin have been described recently (Mackaness and Dodson, 1957).

RESULTS

The effect of "foetal ablation" on the blood pressure of pregnant hypertensive rats

When a group of rats had become frankly hypertensive, about six weeks after placing a clip on the left renal arteries, they were mated and their blood pressures recorded every day or second day. Of 20 rats only six became pregnant. Their mean blood pressure was 173 mm. Hg at the time of conception. The date of conception was estimated from the presence of a copulation plug if one was found; otherwise it was estimated by abdominal palpation as soon as the conceptuses could be felt. The foetuses were removed on the 14th to 17th day of pregnancy and their average weight gave a further estimate of the stage of pregnancy. Blood pressures were recorded until it was considered that the animals had completely recovered from the effects of their operation and pregnancy. It was considered that such was the case 33 days after conception; that is about 12–13 days *post partum*. Hysterectomy was then carried out on each member of the group, and blood pressures recorded for another 5 days.

The blood pressures of the rats which had undergone foetal ablation followed the usual course observed in hypertensive rats during pregnancy. The pressures, which had started to fall when the foetuses were removed, continued to do so after the operation and rose abruptly about the expected date of term. The mean pressures of these rats during the experiment have been plotted in Fig. 1. For comparison Grollman's data (1949) for the mean blood pressure of a group of 12 hypertensive rats taken at intervals through pregnancy has been plotted on the same scale.

The effect of "total ablation" on the blood pressure of hypertensive rats

The operation of total ablation—that is the removal of the placentas as well as the foetuses—was carried out on a group of six hypertensive rats between the 14th and 18th day of pregnancy. This operation is very similar to that of foetal ablation and, if anything, is likely to cause more operative shock. Post-operatively the blood pressures of the group were recorded for another 10 days.

At the time of ablation, between the 14th and 18th day of pregnancy, the blood pressures of the rats were distinctly below their pre-pregnant hypertensive levels. Immediately after the operation the blood pressures commenced

to rise and were substantially higher on the second post-operative day. In Fig. 2 a comparison is made of the changes in blood pressure produced by total and foetal ablation. It will be seen that the blood pressures of rats which had undergone total ablation rose progressively until the pre-pregnant level was reached on the fourth post-operative day. In contrast rats from which only the foetuses had been removed showed a progressive fall in blood pressure during these 4 days.

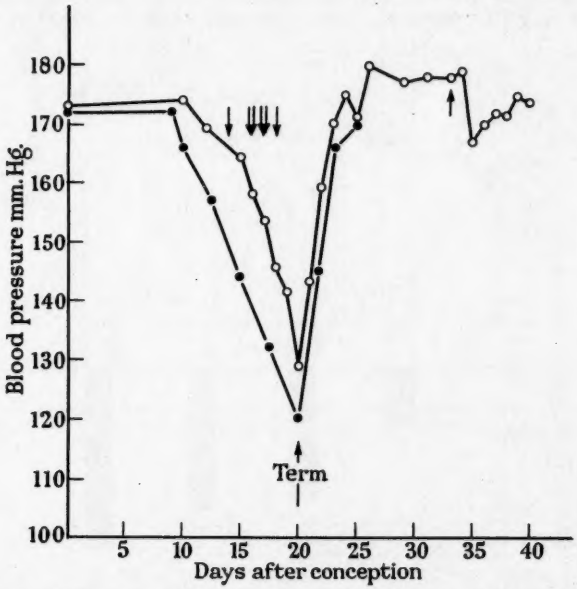

FIG. 1.—The effect of foetal ablation on the blood pressures of hypertensive rats. (Solid circles) ●——● (Grollman's data)—the mean B.P. of 12 hypertensive rats during pregnancy. (Open circles) ○——○ the mean blood pressure of 6 hypertensive pregnant rats undergoing foetal ablation ↓ and hysterectomy ↑.

The effect of "foetal ablation" on the pressor response of pregnant rats to renin

The foetuses were removed from a group of 11 normal rats between the 13th and 16th day of pregnancy. Three to five days later intraperitoneal injections of renin were given. The dose was that which produced between 50 and 60 mm. Hg rise in the blood pressure of normal rats after intraperitoneal injection. The renin injections were repeated about every second day until the 6th day after the estimated date of term. Since a reduction of the pressor response to renin may conceivably have resulted from operative shock, the effect of hysterectomy and total ablation on the pressor response was determined.

The mean pressor responses to renin of the group which underwent foetal ablation progressively declined until the expected date of term and rose steadily thereafter. All the post-terminal blood pressure rises were larger than the pre-terminal ones (Fig. 3).

The pressor responses given by the two control groups may be seen in Fig. 4. Clearly, neither hysterectomy nor total ablation interfered significantly with the rat's capacity to respond to renin.

DISCUSSION

The present experiment fully confirms the observation by Page (1947) that the decline in hypertension during late pregnancy is not prevented by excising the foetus and leaving the placenta. It is believed that the controls used in these

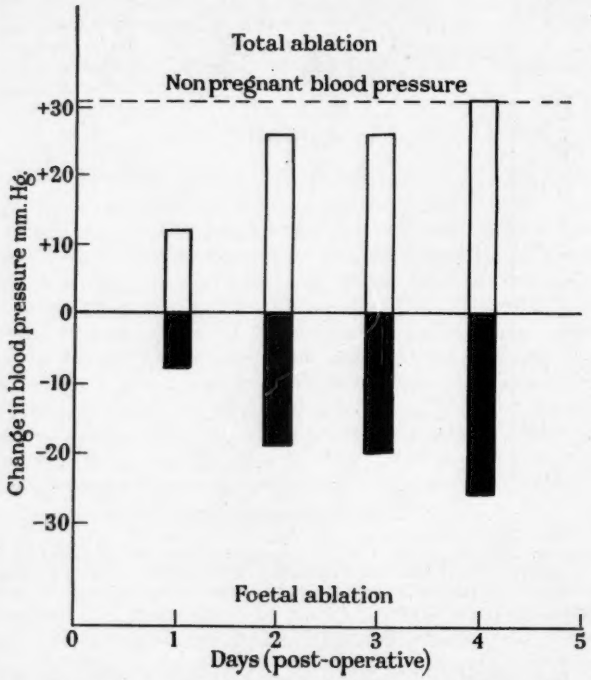

FIG. 2.—Comparison of mean blood pressure changes produced by foetal ablation and total ablation.

experiments meet the criticism that the fall in blood pressure in the animals undergoing foetal ablation was caused by operative shock. It should be noted that the hysterectomies were carried out in the same animals that underwent ablation, and thus a number of variables were excluded. Moreover, the hysterectomies were complicated by the after-effects of the previous operation and under these circumstances hysterectomy was at least as formidable a surgical procedure as foetal ablation. The operation of total ablation, of course, more closely parallels the degree of operative trauma, yet the blood pressures of these rats rose rather than fell post-operatively.

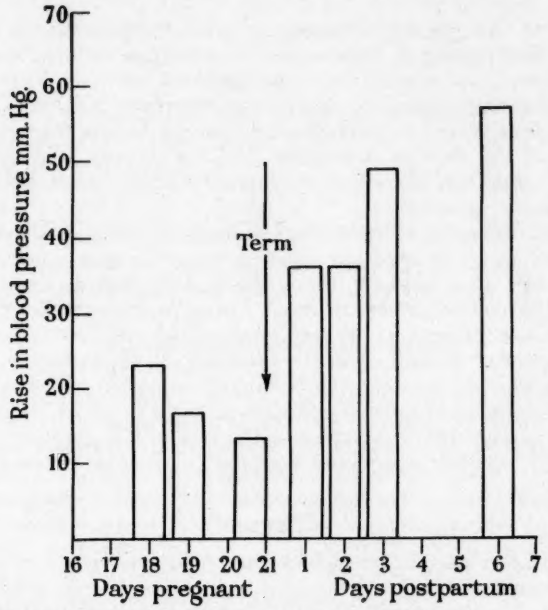

FIG. 3.—Pre- and post-terminal mean blood pressure rises produced by the intraperitoneal injection of renin into rats which had undergone foetal ablation between the 13th and 16th day of pregnancy.

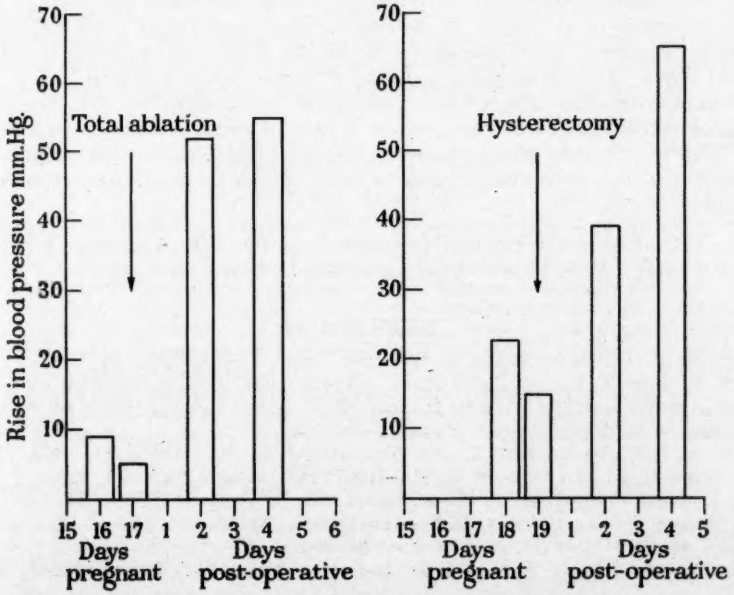

FIG. 4.—Blood pressure rises produced by the intraperitoneal injection of renin in pregnant rats before and after total ablation (left) and hysterectomy (right).

A number of the placentas remaining after foetal ablation were recovered after delivery, and examined histologically. Although necrosis and degeneration were usually found, many chorionic cells appeared normal. This was consistent with the view that they remain functional up to the time of delivery. The placentas were not always delivered on a single day and, doubtless, there was error in the determination of the date of conception and the day on which delivery should have occurred. This had the effect of blurring the sharpness of the terminal dip in pressor response to renin.

The pressor responses of individual animals to renin, as well as the mean responses of the group, showed the same pattern of a steady decline to term and a subsequent rise. It is unlikely, therefore, that the results were due to chance.

It is clear that refractoriness to renin during pregnancy, like the fall in blood pressure, does not depend upon the presence of the foetus; moreover both phenomena appear to depend upon the presence of the placenta. There is, therefore, a close parallelism between the behaviour of hypertensive rats during pregnancy and the response of normal pregnant animals to renin. It is not at present known whether some specific product of the placenta is responsible for the antipressor effect or whether pregnancy produced it in some other way.

SUMMARY

During the latter part of pregnancy the blood pressure of renal hypertensive rats returns to normal levels. It has been confirmed that surgical removal of the foetus without damage to the placenta (which remains functional until term) does not interfere with this fall in blood pressure. Removal of the placentas as well as the foetuses from the gravid uterus causes the blood pressure to return rapidly to hypertensive levels. The foetus (and in particular the foetal kidneys), cannot therefore be the cause of the fall in blood pressure in pregnant hypertensive rats. Moreover, the continuation of the fall after foetal ablation is not due to surgical shock. It is dependent upon the presence of the placenta.

Normotensive rats which have undergone foetal ablation, continue to show the same refractoriness to the pressor action of renin as unoperated pregnant rats. This is a further parallel between the fall of blood pressure in hypertensive pregnant rats and the refractoriness to renin shown by normotensive rats during pregnancy.

The author wishes to express his gratitude to Dr. G. B. Mackaness for the renin used and to Mr. A. B. Howkins for his skilled technical assistance.

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A STUDY OF EXPERIMENTAL BOWEL SHOCK IN RATS. THE INFLUENCE OF VARIOUS AGENTS ON SURVIVAL AND BIOCHEMICAL CHANGES

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GROSS morphological changes in the intestinal mucosa brought about by intestinal strangulation have been extensively studied by Blain and Kennedy (1946), Poth and McClure (1950), Nemir, Hawthorne and Drabkin (1957), Cohn (1956), Cohn and Hawthorne (1951) and Cohn, Gelb and Hawthorne (1953). These authors have established that the changes are caused by the invasion of bacteria and subsequent breakdown of the tissues. The bacteria produce toxic compounds which are rapidly carried to the liver for de-toxification. In more advanced cases the resultant ulcerative necrosis leaves the peritoneal cavity open to the invasion by the intraluminal contents leading to acute peritonitis.

Nelson and Seligson (1953) reported that the level of blood ammonia is increased during and following hæmorrhagic shock in the dog. Also, Engel and Engel (1946) demonstrated that the urea level is decreased during hæmorrhagic shock in the nephrectomized rat.

The purpose of the present study was to determine the survival time and biochemical changes occurring in rats subjected to a standardized bowel shock procedure, and to evaluate the therapeutic effects of various agents.

MATERIALS AND METHODS

Strangulation Obstruction

Healthy, adult male rats, weighing 200–350 g. were maintained *ad libitum* on Purina laboratory chow for a period of at least 2 weeks prior to experimentation.

Food was removed 16 hr. prior to obstruction. The animals were anesthetized with ether. Under aseptic conditions a mid-abdominal incision was made through the linea alba to minimize bleeding. Slip-knot sutures were placed about the circumference of the ileal gut wall in 2 places, 5–7 cm. apart, and tightened. The mesenteric blood supply to the sutured area was occluded in the same manner. The incision was closed and a light coat collodion was swabbed over the sutured surfaces.

The length of time that the obstruction remained varied from 2 to 4 hr., when the peritoneal cavity was again aseptically entered and the slip-knots released. The peritoneum was closed and sutured with cat-gut, while the skin flaps were closed and fastened with stainless steel clamps.

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Pre-treatment

Group 1. Neomycin sulphate, 2·0 g. in 5 ml. of water was administered by stomach tube once daily for a period of 3 days prior to the day of the operation.

Group 2. dl-Methionine, 0·1 g. in 1·0 ml. of water per 100 g. body weight was administered by stomach tube 48, 24, and ½ hr. prior to operation.

Group 3. Neomycin sulphate and dl-Methionine together (as in Groups 1 and 2).

Group 4. A mixture of Purina laboratory chow (13·5 g.); Permutit T (1·5 g.); Bentonite magma suspension (3·8 ml. containing 190 mg. of bentonite); homogenized in a Waring blender was administered to each animal, daily.

Group 5. Chlorpromazine ("Thorazine", Smith, Kline and Fench), intraperitoneally, 5, 10, or 20 mg./kg., 24 and 1 hr. prior to the surgical procedure.

Blood samples

All blood samples were obtained during life by heart puncture. Prior to obstruction 1 ml. of blood was withdrawn from each animal. Immediately after, and again at 1, 2, 3 and 4 hr. following the release of the obstruction a further 1 ml. sample was withdrawn. The samples were heparinized, the red cells removed by centrifugation at 4° and stored at 0°. Whenever a blood sample was taken an equivalent quantity of dextran sulphate was administered by way of the tail vein to keep the plasma volume constant.

Analytical methods

Ammonia was determined immediately on the whole blood employing the Conway micro-diffusion method (Conway, 1947). Plasma urea nitrogen was determined within 12 hr. by the colorimetric method of Gentzkow (1942).

RESULTS

In order to compare the therapeutic effects of various agents in the strangulation obstruction of the small bowel, a standard method of producing the obstruction, shock, and death had to be devised. The influence of duration of obstruction on survival time is summarized in Table I.

TABLE I.—*Influence of Duration of Obstruction on Survival Time*

Obstruction time (hr.)	Survival time (hr.)		No. in Group	Lived	Died	Per cent survival
	Range	Average				
2	102–indef.	Indef.	8	7	1	87·5
3	76–96	86	8	0	8	0·0
4	18–28	23	37	2	35	5·4

Two hours after occlusion the bowel segment appeared unaltered and relatively undistended. Bowel segments occluded for 3 hr. had developed myriads of petechial hæmorrhages throughout the obstructed area, were slightly distended and greenish in appearance.

After 4 hr. occlusion, the bowel segments were turgid with gas, exhibiting a marked degree of intraluminal pressure (Fig. 1) and were bluish-purple in colour. When the slip-knots were released from the occluded segments the intraluminal contents were seen to flow, some leaking into the peritoneal cavity through the necrotized areas.

Within 8–12 hr. after the release of a segment of bowel which had been obstructed for 4 hr. there appeared crusts of blood about the eyes, a foamy exudate from the nose, and bloody diarrhœa. The animals appeared lethargic but

on stimulation became convulsive for periods up to 30 sec. before dying from complete vasomotor collapse (Thomas, 1954).

Effect of Treatment on Standard Strangulation Obstruction

Neomycin sulphate, Permutit T, and chlorpromazine (at the level of 20 mg./kg.) afforded maximal protection against the deleterious effects of the strangulation obstruction. On the other hand methionine seemed to enhance the lethality observed in the control series (Table II). Neomycin sulphate afforded a marked degree of protection even when simultaneously administered with methionine.

TABLE II.—*Survival Time Following Various Pre-treatments*

Series	Obstruction time (hr.)	Survival time (hr.) Range	Survival time (hr.) Average	No. in Group	Lived	Died	Per cent survival	Difference from 4-hr. control (chi square)	Probability of independence from control
Control	4	18–28	23	37	2	35	5·4	—	—
Methionine-treated	4	8–18	12	10	0	10	0·0	0·02	n.s.
Neomycin-treated	4	Indef.	Indef.	25	24	1	96·0	46·64	$P<0·001$
Permutit-T-treated	4	76–Indef.	„	42	37	5	88·0	50·55	$P<0·001$
Chlorpromazine-treated	4								
5 mg./kg.	—	90–Indef.	„	6	2	4	33·3	2·19	n.s.
10 mg./kg.	—	„		6	4	2	66·7	11·44	$P<0·001$
20 mg./kg.	—	Indef.	„	20	18	2	90·0	37·16	$P<0·001$
Methionine + neomycin-treated	4	„	„	8	7	1	87·5	22·81	$P<0·001$

n.s. Not significantly different from control.

The morphological changes observed in the control series were strikingly different from those seen in pre-treated animals (Fig. 2, 3 and 4). Neomycin sulphate and Permutit-T-treated animals had little or no alteration in bowel structure. The Permutit-T-treated animals showed some distension, but it appeared to be of little consequence. The chlorpromazine-treated (20 mg./kg.) rats showed some alteration as evidenced by the greenish-blue appearance of the bowel prior to release.

Animals pre-treated with methionine were similar to the controls in appearance, whereas the methionine plus neomycin sulphate pre-treated animals appeared the same as those treated with neomycin sulphate alone.

Biochemical Changes

The animals employed in this phase of the study were not the same as those used in the experiments to determine survival time. The results of the determinations of blood ammonia and urea nitrogen are presented in Tables III and IV.

DISCUSSION

The closed intestinal loop, employed first by Stone, Bernheim and Whipple (1912) to investigate the formation of toxic proteoses in bowel obstruction of the dog, has become an instrument of great value in demonstrating the local damaging effect of increased intraluminal pressure and the physiological and biochemical consequences of the obstruction.

TABLE III.—*Mean Serum Ammonia and Urea Nitrogen Values Following Release of Obstruction*

Series	Time, hr.	NH_3, μg./100 ml.	Urea nitrogen mg. per cent
Control (16)	Initial	28	19·18
	0	22	42·43
	1	27	39·10
	2	32	31·58
	3	56	21·16
	4	82	18·31
Methionine-treated (10)	Initial	26	20·78
	0	28	27·08
	1	30	28·17
	2	41	24·63
	3	70	20·14
	4	95	17·50
Neomycin-treated (16)	Initial	12	17·02
	0	15	25·52
	1	17	23·43
	2	23	33·37
	3	28	40·81
	4	38	48·90
Permutit-T-treated (8)	Initial	16	21·71
	0	18	26·44
	1	26	33·16
	2	28	38·41
	3	34	42·81
	4	42	52·35
Chlorpromazine-treated (8) (20 mg./kg.)	Initial	27	16·13
	0	29	19·22
	1	33	29·14
	2	41	42·71
	3	43	65·31
	4	52	92·38

Numbers enclosed in () refer to the number of animals employed in the series.

In the present study occlusion of a 5 cm. segment of the ileal region of the intestine for a period of 4 hr., in which mechanical trauma to the tissue was reduced to a minimum, gave highly reproducible states of shock and eventual death.

The therapeutic value of chlorpromazine pre-treatment upon survival following hæmorrhagic and traumatic shock (Hershey *et al.*, 1955; Carruthers and Gowdey, 1956; Jones and Ripstein, 1957) has been confirmed in the present experiments.

That bacteria multiply in obstructed intestinal loops has been adequately demonstrated by MacNeal and Chase (1913), Gork (1922), and Meleney, Jobling, and Berg (1927). Most authors (Sarnoff and Poth, 1944; Sarnoff and Fine, 1945; Schweinburg, Frank, Segel and Fine, 1947; and Poth, 1952) stress the reduction

EXPLANATION OF PLATE

All figures show bowels of rats variously treated after 4 hr. of obstruction.

FIG. 1.—Untreated.
FIG. 2.—Neomycin-treated.
FIG. 3.—Permutit-T-treated.
FIG. 4.—Chlorpromazine-treated.

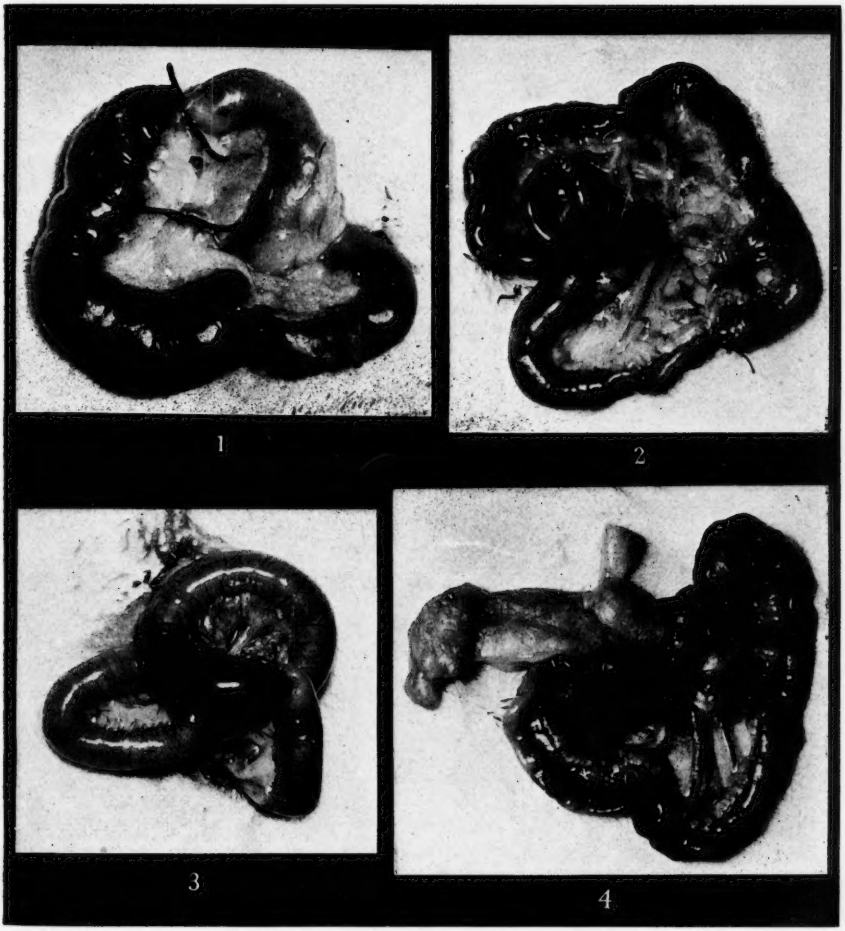

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TABLE IV.—*NH_3 and Urea Nitrogen Determinations Initially and 4 Hr. After Release of Obstruction*

Determination and group	Number of animals	Mean	Range	Probability of significant difference from control
Initial Determination				
NH_3, μg./100 ml.—				
Control	16	27·88	26–31	*
Methionine	10	26·30	25–28	*
Neomycin	16	12·31	8–14	$P<0·001$
Permutit T	8	16·00	15–17	$P<0·001$
Chlorpromazine	8	27·00	23–31	*
Urea nitrogen (mg. per cent)—				
Control	16	19·12	17·05–22·16	*
Methionine	10	20·82	18·28–23·17	*
Neomycin	16	17·07	15·11–19·21	$P<0·001$
Permutit T	8	21·68	19·48–23·36	*
Chlorpromazine	8	16·13	15·15–17·40	$P<0·001$
Fourth Hour Determination				
NH_3, μg./100 ml.—				
Control	16	82·00	80–84	—
Methionine	10	95·30	90–101	$P<0·001$
Neomycin	16	38·00	33–43	$P<0·001$
Permutit T	8	42·00	38–46	$P<0·001$
Chlorpromazine	8	52·00	46–58	$P<0·001$
Urea nitrogen (mg. per cent)—				
Control	16	18·29	16·09–20·52	—
Methionine	10	17·52	16·07–19·03	n.s.
Neomycin	16	48·85	41·51–51·79	$P<0·001$
Permutit T	8	52·32	48·72–54·06	$P<0·001$
Chlorpromazine	8	92·40	89·14–95·72	$P<0·001$

* Within normal range.

of intestinal flora by pre-treatment with intestinal antibiotics as an important factor in obviating necrosis of the bowel wall.

In keeping with the findings of Cohn *et al.* (1953) in the dog, it was demonstrated that pre-treatment with neomycin reduced the lethality and diminished both distention and subsequent tissue necrosis as compared with control animals.

Whether the bacteria themselves, or the products of their metabolism, play the dominant role in the lethality of the animal has been a problem for some time. In studies conducted by Martin and Wilkinson (1947), it has been shown that absorptives such as Permutit T and bentonite are capable of absorbing intestinal toxic products and bacterial lysates. In the present feeding studies it was found that a significant increase in survival occurred if the animals were fed Permutit T and bentonite as 10 per cent of the diet for 3 days prior to occlusion (Table II).

A relationship was observed between survival and the changes in the ammonia and urea nitrogen levels in the peripheral blood system subsequent to strangulation obstruction (Tables II and III). Whether this ammonia load is responsible for the lethality is difficult to assess; however, it appears significant that, although the ammonia concentration in the neomycin-treated animals following strangulation obstruction is higher than that of normal animals, the urea synthesis is also increased. On the other hand, urea synthesis in unprotected animals diminishes

while the ammonia concentration continues to increase (Table III). This observation suggests that the end-products of protein decomposition may play an important role in precipitating death.

It was observed that methionine enhances lethality following strangulation obstruction. A possible explanation of the deleterious effects of methionine based on the work of Rosenbaum, Frank, Rutenberg and Frank (1957), who have demonstrated that the decline of high energy phosphate content of the liver during hæmorrhagic shock is reduced by prior treatment with aureomycin, might be as follows: various autolytic breakdown products resulting from bacterial action within the gut wall may damage the liver to the point where it is no longer able to detoxify these noxious products because of a depletion in the high energy phosphate content. Bacterial action on the administered methionine might be expected to give rise to compounds such as methione sulphoxide or methionine sulphoximine, which would act as antimetabolites in essential reactions (Waelsch, Owades, Miller and Borék, 1946; Schlenk and DePalma, 1955; and Rabinovitz, Olsen and Greenberg, 1956).

SUMMARY

A simple, reproducible method for the production of strangulation obstruction shock in rats was devised. The method consisted of ligation of a 5- to 7-cm. segment of the ileum for a period of 4 hr. This procedure was lethal in 95 per cent of the animals within approximately 18 hr. of release of the obstruction.

Prior to death the animals became lethargic; bloody seepages appeared about the eyes, nose, and anus. Post mortem, numerous petechial hæmorrhages were seen in the lungs, thymus, liver, and gastrointestinal tract.

Determination of blood ammonia and urea nitrogen in the unprotected shocked rats showed a marked rise in the ammonia concentration; the urea level decreased.

Administration of neomycin sulphate, Permutit T, or chlorpromazine at a level of 20 mg./kg. prior to the strangulation obstruction procedure protected the animal against the lethal consequences of the shock. The morphology of the internal organs was normal. The blood ammonia level was almost as high as in unprotected animals, but the urea level showed a concomitant increase.

Methionine was found to enhance the lethal effect of strangulation obstruction. Neomycin sulphate was as effective in protecting methionine-treated animals, as it was in the controls.

Possible mechanisms of these phenomena are discussed.

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The opinions expressed are those of the authors and do not necessarily represent those of any governmental agency.

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A HISTOCHEMICAL STUDY OF WOUND HEALING IN SCORBUTIC GUINEA-PIGS

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AVAILABLE histological evidence indicates that there is little if any reparative activity in the wounds of scorbutic guinea-pigs (Wolbach, 1933). Biochemical evidence shows that the uptake of ^{35}S sulphate by the granulation tissue is reduced in scorbutic guinea-pigs (Kodicek and Loewi, 1955; Upton and Odell, 1956; Moltke, 1957). Histochemical studies of the repair of wounds in such animals yielded controversial results, as far as the presence of acid-polysaccharides is concerned (Penney and Balfour, 1949; Bunting and White, 1950; Bradfield and Kodicek, 1951; Persson, 1953). The present paper deals with a study of the changes occurring during the healing of wounds in scorbutic guinea-pigs by a new histochemical technique (Wolman, 1956).

MATERIALS AND METHODS

Twelve guinea-pigs weighing 250–300 g. were divided into 3 groups of four. Animals of two groups (S and SC) were given a scorbutogenic diet consisting of autoclaved (40 min. at 120°) Purina Laboratory Chow *ad lib.* Twenty-four hours before being sacrificed, the animals of group SC were given 50 mg., of ascorbic acid intramuscularly, whereas the animals of group S were kept in the scorbutic régime. Animals of the control group (C) were given unheated Purina Chow supplemented by fresh vegetables.

A fortnight after the dietary régime was begun all the animals underwent the first operation. The skin over the right scapula was depilated with barium sulphide and a transverse cut, about 12 mm. in length, was made with aseptic precautions. Two days later, another cut was made over the left scapula. Four and 6 days later (18 and 20 days after the dietary regime was begun) similar wounds were made in the two gluteal regions. The wounds which extended into the underlying fascia and muscle were sutured. Two days after the last wounds were inflicted, the animals were killed by bleeding under ether anaesthesia. The wound areas were excised, fixed for 24 hr. in Carnoy's fluid and paraffin blocks were prepared. Sections 6 μ thick were stained with haematoxylin and eosin by the PAS procedure and by the bi-col method. The last-mentioned procedure serves to differentiate weakly acidic tissue constituents (such as hyaluronic acid) which stain red, from strongly acidic ones, such as chondroitin sulphate, DNA, *etc.*, which stain blue (Wolman, 1956).

RESULTS

Normal Guinea-pigs (Group C)

Epithelial regeneration was evident in the 4-day-old wounds and complete by the 6th day. The defect in the 2 days' wound contained an eosinophilic amorphous material which stained intensely with the PAS method and stained pale blue with bi-col. This material was continuous at this stage with a substance of similar staining characteristics which appeared to infiltrate the interstices of the paravulneral tissue. (The designation "paravulneral area" was given to the area

adjacent to the wound and not more than about 1–2 mm. from it.) At 4 days the defect was still well evident and contained a band of eosinophilic fibrinoid material which stained intensely with PAS and very pale blue with bi-col. At 6 days the defect was still not completely filled, but at 8 days the organization was complete.

Fibroblastic and capillary proliferation was observed first in the 4 days' wound; by the 8th day the granulation tissue appeared mature with well oriented capillaries, fibroblasts and collagen fibres.

Ground substance in the paravulneral area of the 2 days' wound appeared imbibed with the fluid which filled the defect. At 4, 6 and 8 days the ground substance near the wound appeared more eosinophilic than that found at a distance.

In the PAS-stained sections no clear-cut differences were found between the ground substance in the neighbourhood of the wound and that situated further away. In the sections stained with bi-col there seemed to be a slight increase of blue and brown-red staining materials in the paravulneral area of the wounds at 4 days. In the 8 days' wounds the paravulneral area contained less blue staining material than in that of the younger wounds and about the same amount as the distant dermis. The amount of the red staining material in the paravulneral area did not decrease with the age of the wounds and was always more than that found further away in the dermis. In the granulation tissue in the dermis and at the cut ends of the muscle, the intercellular material appeared as a weakly stained amorphous mass containing few granules and fibrillary deposits. The amorphous material stained weakly with PAS and pale blue with bi-col. The granules and fibrils stained pink with PAS and either red or blue with bi-col.

Granules which stained intensely blue with bi-col were found within some cells in the granulation tissue. They were few at 4 days and numerous in the 6 and 8 days' wounds. In most animals the muscle near the cut edge stained more intensely blue with bi-col than further away.

Pre-existing collagen fibres were more eosinophilic in the paravulneral area than in the more distant portions as from the 6th day after the incision.

In the muscle, hypertrophy of sarcolemmal cells was noted in the 2 days' wound, and proliferation with formation of giant cells was observed as from the 4th–6th day.

Scorbutic Guinea-pigs (Group S)

Epithelial regeneration occurred at about the same time as in the normal animals. The defect was not filled by granulation tissue even in the 8 days' wound. It countained an amorphous substance which stained weakly with PAS and pale red with or without a faint bluish hue with bi-col. Within the defect in the 4, 6 and 8 days' wounds a band of eosinophilic fibrinoid material was observed. This band which stained with PAS and weakly blue with bi-col, persisted in this group also in the 8 days' wounds.

Fibroblastic and capillary proliferation was absent or minimal in the wounds. Wherever such slight proliferative activity occurred there was complete lack of polarity.

Ground substance in the paravulneral area of the 2 days' wound appeared continuous with the fluid within the defect. In the 4, 6, and 8 days' wounds this area appeared less eosinophilic and more oedematous than that more distant from the wound. The ground substance stained more intensely red with bi-col and with

PAS than the ground substance more distant from the wound, but there was no paravulneral increase of blue-staining material In the ground substance of the paravulneral area of the scorbutic animals there was more red and less blue material with the bi-col stain, and more PAS positive material than in the control animals.

The ground substance between the fibroblasts stained pale red with bi-col, and in the 8-day-old wounds there was also some blue material. The material which stained red with bi-col was more abundant in this group than in the granulation tissue of the control animals, while the opposite was true for the blue-staining material.

Granules within the fibroblasts and histiocytes which stained blue with bi-col were seen only in the 6 and 8 days' wounds.

Collagen fibres near the 6- and 8-day-old wounds appeared hollow in their axial parts, *i.e.*, less eosinophilic.

No appreciable differences were noted in the sarcolemmal reaction of these animals as compared with the normal group.

The dermis of these animals far from the wounds appeared of a more homogeneous texture than that of normal guinea-pigs. The ground substance stained more intensely red and slightly less blue with bi-col than that of the normal animals, and more intensely with PAS.

Scorbutic Guinea-pigs Terminally Treated with Vitamin C (Group SC)

Epithelial regeneration occurred as in the normal animals. At 2 days the defect and its contents were similar to those of the scorbutic animals. In the 4 days' wound the defect was filled with eosinophilic material which stained more intensely blue with bi-col than in either of the comparable wounds of the two other groups. In the 6- and 8-day old wounds the defect was filled in part and the fibrinoid material was broken into fragments.

Fibroblastic and capillary proliferation was not noted in the 2 and 4 days' wounds. At 6 days there was evidence of active-oriented proliferation and in the 8 days' wound there was a wide band of granulation tissue.

Ground substance in the paravulneral area of the 2 days' wound was similar to that of the scorbutic animals. Around the 4, 6 and 8 days' wounds the ground substance stained slightly more blue with bi-col than in the corresponding wounds of either of the two other groups. The amount of red staining material was slightly less than in the corresponding areas of the scorbutic animals, and definitely more than in the controls. The intensity of staining of this material with PAS was similar to that observed in the scorbutic animals.

Within the granulation tissue the ground substance stained with bi-col pale red with bluish patches.

Numerous intra- and extracellular granules were found around wounds of 4, 6, and 8 days. Almost all of the granules stained deep-blue with bi-col. They were not more numerous than the granules of the scorbutic animals, and their staining with PAS was variable.

The dermis at a distance from the wounds appeared to stain more intensely red with bi-col than in the control group. The amount of PAS-positive material appeared to be less than in the scorbutic animals and more than in the normal ones.

The histochemical findings are tabulated in Table I.

TABLE.—*Comparison Between the Intensity of Histochemical Reactions in Control Animals (C), Scorbutic Animals (S), and Scorbutic Animals Terminally Treated with Vitamin C (SC)*

	Days after wounding											
	Group C				Group S				Group SC			
	2	4	6	8	2	4	6	8	2	4	6	8
Normal skin—												
Bi-col: blue* material.	+	+	+	+	+	±	+	±	+	+	++	±
red† „ .	+	+	++	+	++	++	++	++	++	++	++	++
PAS-positive „ .	±	±	±	±	+	±	±	±	±	±	±	±
Paravulneral area—												
Bi-col: blue* material.	++	++	+	+	+	±	±	±	+	++	++	±
red† „ .	++	++	++	++	++	+++	++++	++++	++	+++	+++	+++
PAS-positive „ .	+	±	±	±	+	++	++	±	+	++	++	++
Granulation tissue ground substance—												
Bi-col: blue material .	..	±	+	++	..	0	+	++§	..	±	+	++§
red „ .	..	+	+	+	..	+	++	+++	..	+	+	++
PAS-positive „ .	..	+	+	±	..	+	+	+	..	+	+	+
Defect area—												
Bi-col: blue material .	+	++	+	0	±	−	−	−	±	±	−	±
red „ .	−	−	+	0	±	+	+	+	+	+	+	+
PAS-positive „ .	++	+	−	0	+	+	+	+	+	+	+	+

* Blue stained material is a strongly acidic polysaccharide.
† Red stained material is a weakly acidic polysaccharide.
§ In group S where little granulation tissue was formed, the data concern the scanty ground substance found among the fibroblasts.
0 = no staining; ± very weak staining; number of + signs indicates intensity of staining reactions.

DISCUSSION

This study corroborates the notion that the disturbed healing of wounds in scorbutic animals is accompanied by (or due to) a diminished sulphation of the polysaccharides of ground substance. In the paravulneral area of scorbutic animals there was more PAS-positive material and more material which stained red with bi-col than in the normal animals. It has been shown that chondroitin sulphate and hyaluronic acid are stained only weakly or not stained at all by the PAS procedure (Davies, 1952); Persson, 1953; Braden, 1955; Hayashi *et al.*, 1955; Hooghwinkel and Smits, 1957). The red staining with bi-col indicates the presence of weak acidic groups such as those of uronic acids; it is obvious, therefore, that the ground substance of the dermis of scorbutic animals contains more non-sulphated polysaccharide than that of normals. This holds true for all the dermis and especially that of the paravulneral area. Whether the increase of the polysaccharide in the scorbutic animals is due to the presence of the recently isolated non-sulphated chondroitin (Davidson and Meyer, 1954) is not clear from this work, as no information regarding the stainability of this compound by the PAS procedure is available. Our findings closely agree with biochemical studies (Persson, 1953; Robertson and Hinds, 1956), which indicated that in scurvy there is an increased production of polysaccharide and diminished sulphate fixation in the granulation tissue and in the paravulneral area.

On the whole, the paravulneral area of scorbutic animals did not appear to contain much less sulphated (*i.e.*, staning blue with bi-col) ground substance than that of normal animals. The difference was more obvious when the amount of this material was correlated with the total amount of polysaccharides. In the granulation tissue itself, however, the difference was more marked : in the scorbutic animals there was very little newly formed tissue and that tissue contained little sulphated material, whereas in the normal animals there was much sulphated ground substance in the abundantly growing granulation tissue.

The findings in the scorbutic guinea-pigs which were given vitamin C 24 hr. before death show that under these conditions the repair processes progress more rapidly than in normal animals (Penney and Balfour, 1949). The administration of vitamin C caused a rapid increase in the amount of sulphated ground substance (mainly in the paravulneral area) without appreciable decrease in the amount of the non-sulphated polysaccharide present, accompanied by a very rapid growth of granulation tissue in the older (6 and 8 days') wounds. The rapid growth of the granulation tissue probably indicates that the necessary materials for the proliferation were formed also in the scorbutic animals, except for the sulphated polysaccharide. The formation of the latter might have allowed a rapid utilization of the prepared materials. These findings are in accord with the data of Campani and Reggianini (1950) who suggested that active sulphation of the " hyaluronic acid " of healing wounds starts about the 6th day, *i.e.*, after the polysaccharide was laid down, and the data of Dunphy and Udupa (1955) who found that a productive phase in which the building blocks of repair are formed precedes the phase of collagen formation.

There are many indications in the literature which explain the possible importance of sulphated polysaccharides in the determination of the orientation of granulation tissue and collagen. The charged molecules of the sulphated polysaccharides might serve as a frame which orientates the micelles of pre-collagen (Cohen, 1942 ; Partridge, 1948 ; Jackson, 1953 ; Enghusen, 1955 ; Loeven, 1955). If this assumption is correct, the free sulphate groups would be of primary importance in the process of wound healing. The bi-col method allows the differentiation of these groups from less charged and uncharged molecular groups and appears to be more dependable than metachromasia. The discrepancy between the results of workers who used the metachromatic staining is probably due to differences in technique. At a high enough pH both the strong and the weak acidic groups will react metachromatically, whereas at a low pH, the uronic groups are not ionized and consequently do not react. Furthermore, alcohol treatment tends to abolish the metachromasia due to the weak acidic groups.

SUMMARY

The process of wound healing was studied by histological and histochemical methods in 3 groups of guinea-pigs : (*a*) scorbutic ; (*b*) scorbutic, terminally treated with ascorbic acid ; (*c*) normal controls.

In the scorbutic animals there was marked increase of weakly acidic and of neutral (PAS reactive) polysaccharides of the ground substance, as compared to the controls. There was a moderate decrease in the amount of the strongly acidic polysaccharides, but a marked decrease in the ratio of strongly acidic to weakly acidic components.

Twenty-four hours after the administration of ascorbic acid to scorbutic animals there was a regression of the relative amounts of the polysaccharides towards the state prevailing in the controls in the 6- and 8-day-old wounds. This was accompanied by an enhanced formation of granulation tissue.

The restorative effect of the ascorbic acid on the older (6 and 8 days) wounds seems to indicate that during the stages of wound healing in the scorbutic state materials necessary for repair are formed but not utilized.

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THE GROWTH AND CYTOPATHOGENICITY OF SHEEP-POX VIRUS IN TISSUE CULTURES

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MANY viruses of the pox group have now been propagated in embryonated eggs or tissue cultures but a few still remain for which no satisfactory experimental host exists. These include the agents of sheep-pox, goat-pox, horse-pox, and, until very recently, contagious ecthyma of sheep and goats (see Abdussalam, 1957*a*; Grieg, 1957).

The present communication describes the adaptation of a single strain of sheep-pox virus to growth in cultures of cells derived from sheep, goats and calves.

MATERIALS AND METHODS

Tissue cultures

Trypsin-dispersed cells were produced from the testes of prepubertal or, rarely, embryonic sheep by a simple modification of Youngner's (1954) technique which has already been described (Ferris and Plowright, 1958). Lambs were usually castrated at about 4 to 10 weeks of age, the foetuses being near-term.

It has recently been found advantageous to extend the period of trypsinisation of testis tissue to 2 hr. instead of about 45 min. After this time the cells sedimented cleanly on centrifugation, without evidence of the mucoid material which was originally noted to cause difficulty. In addition, a larger yield of cells was obtained and 400 to 800 ml. of cell suspension could be prepared from the testes of a single lamb. Calf testis monolayers were prepared as previously described (Ferris and Plowright, 1958) utilising 2 hr. period of trypsinisation.

Sheep testis cell suspensions were standardised to contain about 2×10^6 isolated cells per ml. of a growth medium consisting of 0·5 per cent lactalbumin hydrolysate (Nutritional Biochemicals Corpn.), 0·1 per cent bacteriological yeast extract (Difco) and 10 per cent normal, unheated ox serum (O.S.) in Hank's saline. Tube cultures (160 × 15 mm.) received 1 ml. of cell suspension and were incubated sloped in a stationary position for 3 or more days. Virus passages were carried out using cultures in 8 cm. Carrel flasks, which were seeded with 10 ml. of cell suspension; Roux flasks received 75 ml., 10 cm. Petri dishes 10 ml.

Sheep kidney-cell monolayers were produced by a method similar to that of Bodian (1956), from the kidneys of lambs and near-term foetuses. For these cells the growth medium was identical to the one described above, except that 5 per cent ox serum was used for cells from sheep embryos. In one instance cells of a 4th *in vitro* subculture of sheep embryonic kidney were employed. Goat kidney-cell suspensions were prepared by Bodian's method from the kidneys of 1–2 month-old kids, 5 per cent O.S. being used in the growth medium.

The medium for all types of culture was changed after 2–4 days to one with Earle's saline, 0·5 per cent lactalbumin hydrolysate and 0·1 per cent yeast extract. This corresponds with the L.A.Y.E. medium of Franklin, Rubin and Davis (1957). Depending on the growth vigour of the cells, 5 or 10 per cent O.S. was added.

Testis cultures and those from embryonic sheep kidneys often formed continuous cell sheets by the 2nd or 3rd day and were sometimes inoculated with virus at this time. Kidney-cell monolayers from lambs and kids were not usually inoculated until the 5th day or later. All cultures were incubated at 36·5 ± 0·5°.

Virus strain

This was obtained from a severe outbreak of sheep-pox which involved Merino-grade lambs in the Rift Valley of Kenya during 1955 (MacOwan, 1956). The source material was a 10 per cent glycerinated skin lesion suspension from an experimental sheep. This was inoculated intradermally into two further adult sheep (Nos. 1 and 2) which showed typical local reactions. A skin lesion suspension from one of these was inoculated intrathoracically into a week-old lamb which developed pyrexia, generalised skin lesions, a characteristic pneumonia and pleurisy with effusion. Pleural fluid was harvested aseptically, heparinised and used to infect cultures.

Infected tissues and fluids were stored frozen at − 20 to − 25°.

Virus passage

Undiluted fluids from previous passages were used for the kidney cultures and for the first 2 testis-cell passages. For later testis passages whole cultures, with the fluid in contact with the cells, were frozen at − 25° on the 5th to 9th day following infection. These frozen monolayers were later thawed, shaken to detach the cells and the resulting suspension ground with sand in a mortar or, latterly, in TenBroeck grinders for 2 min. (Overman and Tamm, 1957).

All virus samples were centrifuged at 2000 r.p.m. for 5 min. to free them from gross particulate material. Of supernatant 1·0 ml. was used to infect 8 cm. Carrel flasks or 10 cm. Petri dishes, 7·5 ml. for Roux flasks. For virus adsorption we allowed ½–2 hr. at 36°, fresh maintenance medium (L.A.Y.E. with 5 per cent O.S.) then being added. Further changes of medium were sometimes made after 4–5 days.

Virus titrations

Virus was prepared in 10-fold dilutions in chilled phosphate buffered saline (P.B.S.) (Dulbecco and Vogt, 1954) or medium without serum. Four serial dilutions of 0·2 ml. quantities were inoculated into four or more tubes of sheep testis cells per dilution and adsorption took place for 1–2 hr. at 36·5° in a roller-drum. Fresh maintenance medium was added and the tubes were incubated for 11 to 12 days, either stationary or returned to the roller-drum. No further fluid changes were done during this time. Low-power microscopic examinations were made at intervals and all tubes showing one or more distinct focal " lesions " were regarded as infected.

The 50 per cent cytopathogenic end-point was calculated using Thompson's method (1947) and results were expressed as the reciprocal of the logarithm of the 50 per cent end-point dilution.

Coverslip preparations of infected cultures

The majority of virus passages and the growth curve studies were combined with the infection of cultures in 10 cm. Petri dishes containing eight ⅞ in. No. I circular coverslips (Ferris and Plowright, 1958). One dish was kept as an uninfected control and coverslips were removed from both control and infected cultures at approximately daily intervals. They were rinsed in saline, fixed for 10–20 min. in Bouin's fluid, decolourised in several changes of 70 per cent alcohol and stained with Ehrlich's haematoxylin and eosin or by Giemsa. The latter was differentiated by a method similar to that of Jacobson and Webb (1952). Giemsa staining was less satisfactory for showing cytoplasmic inclusions or nuclear changes and its use was soon discontinued.

Sheep inoculations

Two types of sheep were employed for virus titrations and pathogenicity studies: (*a*) Lambs 5–6 months old with at least 50 per cent Merino blood (*b*) Lambs 2–3 months old of indigenous African fat-tailed type, with a hairy fleece.

Titrations were carried out by intradermal inoculation of 0·2 ml. quantities of serial dilutions into the shaved skin of the flank. Each dilution was inoculated at 5 sites about 1·5 to 2·0 cm. apart. The animals were examined frequently over 28 days and the temperature recorded daily. Challenge of immunity was by intradermal inoculation of 0·1 ml., at five sites on the opposite flank, of undiluted pleural fluid from the lamb which served as the original source of the culture virus.

Virus-neutralising antibody tests

Pre- and post-inoculation sera were collected from experimental sheep and stored at — 25°. Two types of neutralisation test were performed.

(*a*) Dilutions of serum 4- or 5-fold in P.B.S. or in medium without serum were mixed with an equal volume of culture-passaged virus estimated to contain about 100 ID_{50} per 0·1 ml. The mixtures were left overnight at 4° and then inoculated, in 0·2 ml. quantities, into 4 or more tubes of sheep testis cells per dilution. Low-power microscopic examination was carried out up to the 10th or 11th day.

(*b*) Because the constant-virus, serum-dilution method gave very low antibody titres, due to late " break-through " of virus, tests were also carried out in which a virus sample was titrated in the presence of equal volumes of undiluted sera, one obtained before infection, one or more during convalescence from each sheep. The mixtures were also left overnight in the refrigerator and inoculated on the following day into sheep testis cultures ; four virus dilutions were employed, with 4 tubes per dilution. The potency of the serum was expressed as a neutralisation index, *i.e.*, the difference between the 50 per cent end-point of the virus when titrated in serum-free medium and in an undiluted serum.

RESULTS

Pleural fluid from a sheep-pox infected lamb produced cytopathic effects in embryonic sheep kidney monolayers, these being detected from the following day onwards. The changes began in rounded foci or tracts which became thickened and opaque. The cells in these areas tended to become rounded and granular or, less frequently, refractile, angular or spindle-shaped, with short spiky processes. These cytopathic effects did not extend to involve the whole cell-sheet, even when cultures were maintained for 3 weeks after infection. Two further kidney cell passages were carried out using undiluted fluids, harvested 11 or 12 days following virus inoculation. The cells in infected monolayers were also trypsinised and subcultured and " clean " cell suspensions were infected and then seeded into vessels in which they formed monolayers. All of these procedures resulted in cytopathic effects visible from the 4th to 6th days but they failed to become generalised ; furthermore very few cells detached, although some thinning of the cell sheet and angular clearings were occasionally seen in the centre of older cytopathic foci. Goat kidney monolayers showed typical focal changes from the 5th day onwards but these also failed to generalise.

EXPLANATION OF PLATES

All figures are of sheep testis monolayers grown on coverslips in Petri dishes. Fixation was by Bouin's fluid, staining by Ehrlich's haematoxylin and eosin.

FIG. 1.—Five-day culture, infected 46 hr. previously with sheep-pox virus. Retraction of cytoplasm and assumption of spindle shape at an early stage. × 200.

FIG. 2.—Six-day culture, infected 3 days. Further separation of cells and spindle cell change. × 200.

FIG. 3.—Seven-day culture, infected 4 days. Thinning of cells. Numerous cytoplasmic inclusions just visible. × 200.

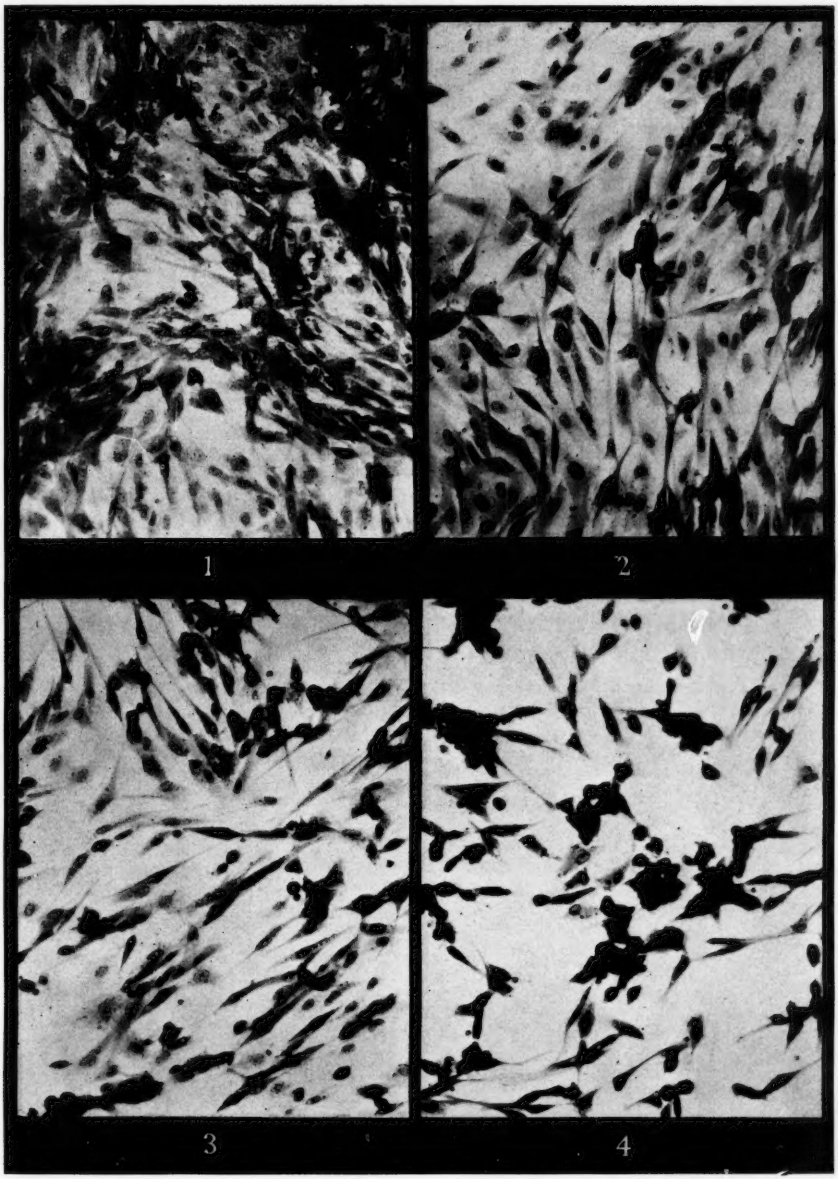
FIG. 4.—Eleven-day culture, 8 days post-infection. Clumping and rounding of cells. Nuclei appear vesicular. × 200.

FIG. 5.—Seven-day uninfected monolayer. Note ill-defined cell boundaries. × 200.

FIG. 6.—Same culture as for Fig. 4. Margination of chromatin and nucleoli, eosinophilic material occupying centre of nuclei. Cytoplasmic inclusions, some with halo. × 720.

FIG. 7.—Same culture as for Fig. 5. High power showing normal nuclear structure. × 1860.

FIG. 8.—Same culture as for Fig. 6. One or more intranuclear foci with accumulation of granular eosinophilic material. Peripheral displacement of chromatin and nucleoli. × 1860.



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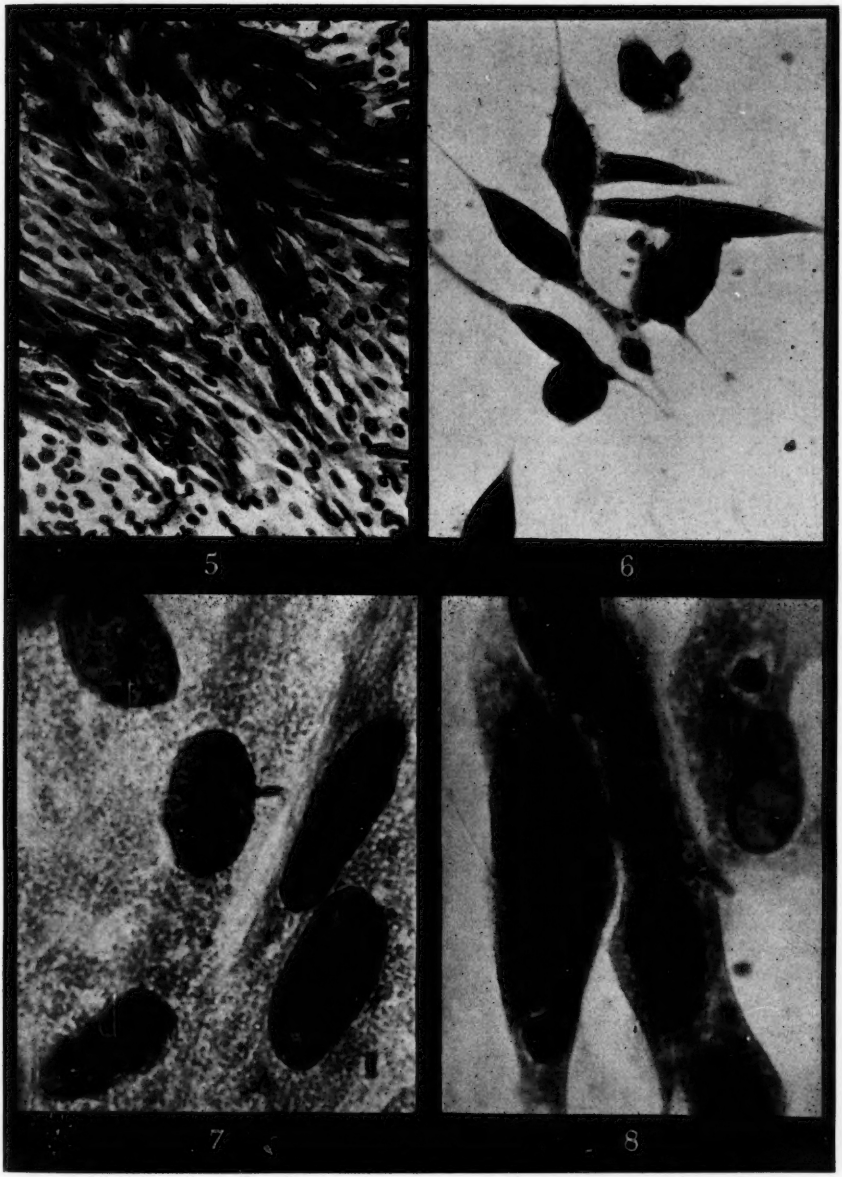

Plowright and Ferris.

From the 2nd sheep kidney passage 12-day fluid was used to initiate a virus line in sheep-testis monolayers. Passage was made 16 times in this cell type, which proved decidedly superior. During the first passage virus-induced changes were noted on the 5th day and practically all cells were affected by the 8th day following infection. In subsequent passages cytopathic effects were clearly visible in unstained cultures from the 2nd day after virus inoculation and were regularly generalised 3 days later.

When virus of the 7th or later testis passage was titrated in tube cultures of the same cell type, it was found that inocula of less than about 100 $I.D._{50}$ resulted in the appearance of discrete greyish or whitish foci of cytopathic change, first detected between the 5th and 11th days. If such cultures were retained, secondary " plaques " or a diffuse change were often visible after a further 3 to 5 days.

Virus from the 10th sheep testis passage was used to initiate a line in calf testis monolayers. Five passages were carried out at intervals of 4–6 days, using frozen, whole-culture material as inoculum. The cytopathogenic effect was very similar in type and rapidity of development to that described for sheep testis cells ; if young (2–3 day) cultures were inoculated practically every cell was involved at the 4th to 6th day.

Growth Curve of Sheep-pox Virus in Testis Fibroblasts

Two experiments were conducted. In one a Roux-flask monolayer and two 8 cm. Carrel-flask cultures were inoculated with virus after removing the medium. After 75 min. at 36° to allow virus adsorption, the cultures were washed 3 times with pre-warmed P.B.S. and fresh medium was added. Aliquots of the fluid in the Roux flask were removed daily and stored at −25° after centrifugation. The Carrel-flask cultures were frozen after 3 and 5 days respectively without prior removal of the fluid. The Roux-flask culture was treated similarly after 8 days. The frozen cultures were thawed immediately before titration, shaken vigorously to detach the cells and triturated for 2 min. in a glass grinder. The resulting suspensions were centrifuged and titrated, together with the stored fluids.

The results are given in Fig. 9 which shows that progeny or eluted virus was detected from the first day onwards ; fluid titres of about 10^5 $I.D._{50}$ per ml. were attained by the 5th and persisted until the 8th day. On the 3rd and 5th days virus in the fluid constituted only 3 to 5 per cent of the total present in the culture. The approximation of total and fluid virus on the 8th day may have been due to cessation of production and heat inactivation, but this was not repeated on the 7th day in the next experiment.

In the second experiment seven uniform Carrel-flask cultures were all inoculated with $10^{5.2}$ T.C. $I.D._{50}$ of 7th passage virus. On each of the following 7 days fluid was harvested from one flask and centrifuged. The monolayer was then washed 3 times with 10 ml. of P.B.S. to remove free virus, 10 ml. of fresh medium was added and the flask plus the separate fluid harvest were both stored at −25° until titrated. For titration of the cell-associated virus, frozen cultures were treated as described in the first experiment. The results are given in Fig. 10, which shows the estimated quantities of virus (*a*) free in the fluid and (*b*) associated with the cells, at various intervals after infection.

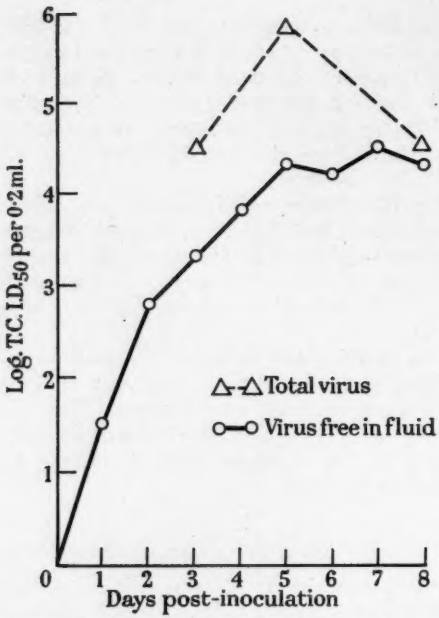

FIG. 9.—Growth of sheep-pox virus in sheep testis monolayer.

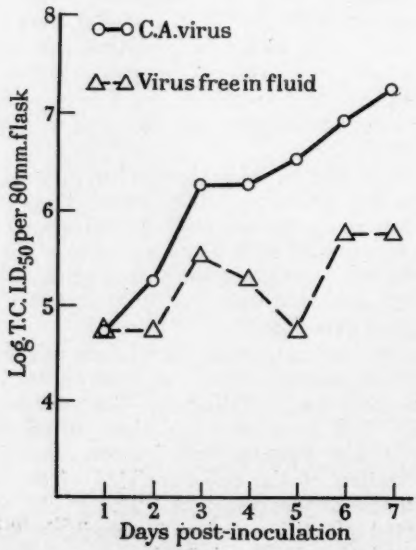

FIG. 10.—Growth of sheep-pox virus in sheep testis monolayers.

After 24 hr. the amount of virus in the fluid and in the cells was identical; the former must be largely accounted for by unadsorbed inoculum. Cell-associated virus (Howes and Melnick, 1957), showed a continuous increase up to the 7th day whilst there was relatively little change in the quantity demonstrated in the fluid. Between the 4th and 7th days the latter varied between about 2 and 10 per cent of the total.

Cytopathic Effects of Sheep-pox Virus

Monolayers of sheep testis fibroblasts inoculated with large doses of virus occasionally showed changes as early as 18–24 hr., but these were not easily detected in unstained cultures. On the 2nd day following infection, the cell arrangement was distinctly irregular in some areas and cell outlines were becoming clearly demarcated, whereas in control cultures they were difficult to distinguish (Fig. 1 and 5). There was retraction of the cytoplasm so that by the 3rd day many cells were spindle-shaped with long pointed extremities. Some of these changes we designated " angular separation ".

There was an early tendency to increasing refractility, later obscured by the accumulation of fine granular material in the cytoplasm. Infected cultures could easily be distinguished from the 3rd day onwards by a whitish opacity visible with the naked eye in transmitted light. The final stages of degeneration were marked by a rounding and clumping of infected cells, first visible at the 3rd day but not a marked feature until the 5th day and later. The cytoplasmic retraction mentioned above resulted in the appearance of angular, cleared areas and these were accentuated by some detachment of infected cells. Nevertheless, many of the latter remained attached to the glass at the 8th day (Fig. 1–4). Some turbidity of the medium was present from about the 3rd day.

The appearance of fixed and stained coverslip preparations, infected in parallel with the cultures used to construct Fig. 9, is depicted in Fig. 1–4, 6 and 8. Cytoplasmic retraction and the assumption of spindle shape was accompanied by an increasing cytoplasmic basophilia. This was sufficiently marked to cause the infected coverslip to be macroscopically a deep purplish-blue with haematoxylin and eosin, as opposed to the light mauve staining of normal controls.

Small neutrophilic or eosinophilic inclusions were present in the cytoplasm of a few cells at 24 hr., their number increasing so that virtually every cell showed one or more of them by the 4th day. These inclusions were at first granular and ill-defined; later, however, they became larger, denser, more basophilic and acquired a well-marked " halo ". They usually occurred towards one pole of the cell, often close to the nucleus. High power examination showed sometimes a suggestion of a limiting membrane (see Fig. 8) and there were often deeply basophilic " inner bodies ". In the rounded, dense granular cells which represented the final stage of degeneration, cytoplasmic inclusions could still be vaguely detected.

Limited observations were made on infected coverslip preparations using high-power, phase-contrast microscopy (× 1600). It was possible to confirm that the halo surrounding the cytoplasmic inclusions was present in the living cell and not therefore a fixation artefact. Within the inclusion small elongated elementary bodies of regular size and shape were distinguishable.

Changes in the nuclei of infected cells were observed from the 3rd day onwards and at the 7th–8th day practically all of them were abnormal (Fig. 6 and 8).

A finely granular or homogenous material, which was frankly eosinophilic, appeared at one or several foci within the normal chromatin network (Fig. 7). The nucleoli and the chromatin were displaced peripherally by accumulation of this material so that finally there was a dense, thickened nuclear membrane on which the nucleoli rested (Fig. 6). There was never any separation of the central eosinophilic mass and the marginated chromatin ; in this respect, therefore, it could not be said that an intranuclear " inclusion " had formed (Cowdry, 1934). Microscopic observations on infected calf testis monolayers showed changes practically identical to those in sheep cells. It was noted, however, that a cleared zone appeared not infrequently around the granular eosinophilic material which accumulated in the nuclei of infected cells.

The cytologic changes which were observed in fixed and stained kidney cells from embryonic sheep, or from lambs or kids, were essentially similar to those in sheep testis fibroblasts. Epithelial cells, however, showed little tendency to become spindle-shaped but did form dense, heaped-up masses of rounded cells. These often showed a heavy accumulation of eosinophilic granules and always typical neutrophilic or basophilic inclusions in their cytoplasm. Nuclear changes were as frequent and as marked as in testis cells. There was very little or no clearing of the glass surface even after periods of up to 3 weeks ; it was confirmed that large tracts of cells remained normal on examination of fixed and stained preparations at this time.

Pathogenicity for Sheep of Culture Passaged Virus

Virus from the 10th testis cell passage, in the form of homogenised whole culture, was titrated simultaneously in the skin of four sheep and in testis cultures using 8 tubes per dilution. The culture titre was 6·3 per 0·2 ml. Each sheep was inoculated at five sites with all dilutions from 10^{-1} to 10^{-7}. The results are given in Table I.

TABLE I.—*Sheep Titrations of 10th Testis-passage Virus*

Sheep No.	Breed	Titre*	Days of temperature reaction	Generalisation	Reaction to challenge
5	Indigenous African	6·7	5–6 incl.	—	*Nil*
6	„ „	6·9	6–8 „	+	„
9	Merino-Grade	7·1	5–9 „	+	N.D.
10	„ „	6·9	4–7 „	+	„

N.D. = Not done.

* Per 0·2 ml., expressed as reciprocal of the log of the 50 per cent end point, calculated by Thompson's method.

Skin lesions appeared at the sites of inoculation from the 2nd to the 8th days post-inoculation depending on the amount of virus ; early ones took the form of raised plaques, about 1 cm. diameter and showing marked erythema when the skin was not pigmented. With larger virus dosage (about 1000 $I.D._{50}$ or more) the plaques showed a spreading oedema which resulted in confluence between the 4th and 7th days. Their centres became purplish, with superficial necrosis and slight serum exudation from the 6th day onwards whilst a thin, dark layer of scab was visible from the 8th day. The scab was tightly adherent and only fell

at the end of the 4th week, leaving areas of underlying granulation tissue still exuding small quantities of serum.

Three of the four sheep showed a few secondary " pocks " on the 8th day. They were detected at one or more of the following sites, *viz.*: axilla, groin, scrotum and under-surface of the tail. They regressed rapidly and were not palpable on the 12th day. The two Merino-Grade sheep died on the 31st day after inoculation, but this was not thought to be directly related to the sheep-pox infection. Both African sheep showed no reaction to challenge on the 42nd day with virulent lymph, whereas an uninoculated Merino which had been maintained throughout in close contact with the infected sheep was found to be still fully susceptible. This latter, incidentally, showed severe local reactions, the lesions having a haemorrhagic centre from the 3rd day and adherent blackish scabs which fell on the 28th day. There was fever on days 4–6 inclusive but no generalisation.

Neutralisation of Culture Virus by Sheep Sera

Unheated sera from Merino-Grade or indigenous sheep later proved susceptible by intradermal inoculation of sheep-pox virus, almost completely neutralised 1000 T.C. $I.D._{50}$ of culture virus. For each of 6 sheep sera, for example, only one tube out of four inoculated showed small focal lesions when inoculated with such serum-virus mixtures. Heating at 56° for 30 min. completely removed this non-specific effect, so that virus titres in the presence of an equal volume of undiluted, heated serum were identical to or higher than those obtained by dilution in a medium without serum (see Table II).

When the sera of convalescent sheep were titrated against a constant quantity of virus, it was found that the cytopathic effect was markedly reduced, in some cases up to a dilution of 1/128 (Table II). On the other hand, one or two small foci of infection still appeared in nearly all tubes inoculated with mixtures containing undiluted serum and 150 $I.D._{50}$ of culture virus. Occasionally, with still smaller quantities of virus (40 $I.D._{50}$), completely negative end-points could be obtained. More precise figures for the relative potency of sera could be obtained by the titration of a virus sample in the presence of undiluted sera. Even by this method it was only possible to show a half to one hundred-fold reduction in the surviving virus fraction (Table II).

DISCUSSION

The majority of investigations of sheep-pox have been primarily concerned with the production of vaccine virus for large-scale immunisation programmes. Rafyi and Mir Chamsy (1956), for example, reported on the immunisation of 20 million sheep in Iran during the 7 years preceding their publication. Vaccine virus has hitherto been produced by the subcutaneous inoculation or dermal scarification of sheep, methods open to serious objections in that the resulting material is difficult to handle, never sterile and always liable to include other sheep pathogens such as that of contagious agalactia (Bridré and Donatien, 1925). In addition, such virus requires to be standardised by titration in the skin of sheep, an expensive and laborious procedure.

Various attempts have therefore been made to adapt sheep-pox virus to growth in embryonated eggs (Gins and Kunert, 1937; Rao, 1938; Ortenzi and Tiecco, 1954; MacOwan, 1956; Sabban, 1957) or rabbits (Bennett, Horgan and Haseeb,

TABLE II.—*Neutralisation of Sheep-pox Virus by Sheep Sera*

Sheep No.	Date of collection of serum	Dilution of serum causing marked reduction of cytopathic effect*	Neutralisation index
1	Preinoc.	*Nil*	0·0
	26†	1/8	−0·3
2	Preinoc.	*Nil*	+0·7
	26	1/32	−1·0
5	Preinoc.	*Nil*	0·0
	14	1/8	−1·5
	21	1/8	−1·0
	28	1/8	N.T.
6	Preinoc.	*Nil*	0·0
	14	1/32	−1·3
	21	1/32	−1·3
	28	1/32	−1·6
9	Preincc.	*Nil*	+0·4
	14	1/8	N.T.
	21	1/128	−2·0
	28	1/128	−1·8
10	Preinoc.	*Nil*	0·0
	14	1/128	−1·7
	21	1/128	−2·2
	28	1/32	−1·8

* Using 150 $I.D._{50}$ of culture virus.
† Figures refer to days post-inoculation

N.T. Not tested.

1944 ; Rao, 1938 ; Sabban, 1957). Only very limited success has been achieved, Sabban being able to transmit the agent through but three direct egg passages.

An early attempt to grow sheep-pox virus in tissue culture was made by Bridré (1935) who carried out four successful passages in suspended fragments of sheep testis. More recently, Rafyi and Mir Chamsy (1956) stated that they had found that the virus would proliferate in " whole tissue " of sheep or guinea-pigs suspended in a suitable medium but that it died out on passage. No proof of virus multiplication was given in either of these instances.

Aygün (1955) reported on the use of plasma-clot, roller-tube cultures of sheep embryonic lung and skin for propagating sheep-pox virus ; he was able to produce an innocuous virus, suitable for the immunisation of Turkish sheep, but failed to observe any cytopathic effect. Greig (1957) was successful in cultivating the agent of contagious ecthyma of sheep and goats in fibroblast monolayers derived from sheep embryonic skin-muscle tissue. He observed marked cytopathic changes which were not described in detail.

Work reported here has shown that monolayers derived from the testes of prepubertal sheep were eminently suitable for the production and titration of sheep-pox virus and may be used for the detection and titration of virus-neutralising antibody. The cells involved were primarily fibroblastic, although sometimes small islets of polygonal cells were observed, these apparently derived from epithelial cell clumps formed by the incomplete breakdown of tubular fragments

during trypsinisation. Kidney cell monolayers from sheep and goats could be infected by the virus and showed specific cytopathic changes, but these remained localised and never involved more than one to two-thirds of the cell sheet. A similar tendency for self-limiting infection has been noted by Noyes (1953), working with chick fibroblast monolayers and vaccinia virus, by several authors with measles virus (Jordan, 1956 ; Ruckle, 1957) and by ourselves with rinderpest and vaccinia in bovine kidney-cell monolayers (Plowright and Ferris, 1957). It may be the result of auto-interference, such as has been postulated by Chambers (1957) for W.E.E. virus growing in " L " strain cells, or to variations in the susceptibility of cells comprising a single culture. In any case virus is released into the fluid but fails to produce cytopathic effects in all the cells thus exposed to infection.

It is interesting to speculate on the possible relationship between differences in the mode of aggregation of some epithelial and fibroblastic cells in culture and the facility with which sheep-pox virus generalises in monolayers of testis as opposed to kidney cells. Using fluorescent antibody techniques and laryngeal carcinoma cells, Noyes and Watson (1955) demonstrated that vaccinia antigens often spread from cell to cell by means of cytoplasmic processes which joined them. The " micro-epidemiology " (Black and Melnick, 1955) of sheep-pox virus in fibroblast cultures may thus depend on the normal existence of cytoplasmic bridges and the absence of a continuous cell membrane, such as delimits each kidney epithelial cell and may cause difficulties in extension of the infection.

Testis cultures fixed and stained at various periods after infection showed changes which could be correlated with the increase of intracellular virus. High titres were maintained from the 4th up to at least the 7th day. The rapid increase of virus during the first four days was accompanied by increase in the numbers and size of cytoplasmic inclusions to the point where virtually every cell contained one or more of these structures. Our limited phase contrast observations on the elongated elementary bodies within the fully formed inclusions are in accord with the electron micrograph studies of Abdussalam (1957*b*) who gives $194 \pm 16{\cdot}08$ mμ for their mean length and $115 \pm 10{\cdot}73$ mμ for the width.

The nuclear changes described were not apparently related to the period of exponential increase of virus ; they appeared later and may be merely of a degenerative character. Similar nuclear changes have already been reported in swinepox (Blakemore and Abdussalam, 1956) and in cultures infected with an agent derived from lumpy-skin disease of cattle (Alexander, Plowright and Haig, 1957). Chapronière (1957) described granules and " vacuoles " in the nuclei of rabbit kidney cells and heart fibroblasts infected with myxoma virus. Downie and Dumbell (1956) quote two descriptions of " intranuclear inclusions" in smallpox and alastrim, but we hesitate to use this term since no distinct formed body was demonstrable in our sheep testis preparations. Similar nuclear changes to those described in culture have been observed by ourselves in skin, lung and liver lesions of experimentally infected lambs and goat kids. We failed, however, to detect cytoplasmic inclusions in the same material, which included skin lesions from animals killed or biopsied 8–14 days after infection.

It is interesting to note that calf testis cells were apparently equally susceptible to culture virus as those derived from sheep in spite of the fact that sheep-pox cannot be transmitted directly to cattle (Rao, 1938 ; Bennett *et al.*, 1944). We inoculated infected sheep pleural fluid intradermally into calves and failed to observe the development of either local or generalised lesions (Plowright and

Ferris, unpublished observations). Scott (personal communication) found that goats were susceptible to this strain of virus and we have confirmed this by inoculation of kids intradermally, intravenously, intrathoracically and by cutaneous scarification. It was not, therefore, surprising that goat kidney cells showed an *in vitro* susceptibility comparable with those from sheep kidneys. Bennett *et al.* (1944) stated that goats did not show any evidence of infection after inoculation of Sudanese sheep-pox strains.

Two passages in kidney and 10 in sheep testis cultures failed to produce any detectable attenuation of sheep-pox virus. On the other hand, contact infection from inoculated to control sheep did not occur with this virus strain either in the instance recorded or in others where susceptible sheep were housed over prolonged periods with infected animals. Scott (personal communication) has made similar observations with the same strain. This failure to spread by simple close contact raises the question as to whether sheep-pox virus, like some other members of the group, may ordinarily require some biting arthropod (either flea, ked, louse, tick or mosquito) for its mechanical transmission.

The difficulty in detecting and titrating neutralising antibody in the sera of sheep convalescent from sheep-pox may be due to the existence of a virus fraction which is relatively difficult to inactivate or cannot be inactivated by antibody. Dulbecco, Vogt and Strickland (1956) showed it to be true with W.E.E and polio viruses and Boulter (1957) has shown that this may be the case with vaccinia. Only plaque reduction methods, such as resorted to by Itoh and Melnick (1957) for Echo 4 virus can give a really satisfactory measure of neutralising activity in circumstances such as those noted for sheep pox.

SUMMARY

An account is given of the adaptation of a single strain of sheep-pox virus to growth in monolayer cultures of sheep, goat and ox cells. In sheep and goat kidney cultures not all cells became affected after prolonged exposure to virus. Testis monolayers derived from sheep and calves showed a complete cytopathic effect of the virus, 16 and 5 passages being carried out in these cell types respectively.

Virus was produced to high titre in sheep testis cultures but 90 per cent or more of the virus remained intracellular, at least up to the 7th day following infection. Peak titres were reached on the 4th to 5th days when large inocula were used; in culture, cytopathogenic end-points were around 10^{-6} per 0·2 ml., whilst in sheep inoculated intradermally titres approached 10^{-7} per 0·2 ml.

The cytopathic changes are described in detail, including the formation of cytoplasmic inclusions and abnormalities in the nuclei which appear to be caused by several of the pox group of viruses. After 12 culture passages there was no demonstrable reduction in the pathogenicity of the strain for sheep. Neutralising antibodies were detected in the sera of convalescent sheep and difficulties in their titration in culture were investigated. Fresh sera from susceptible sheep showed marked inhibitory activity against culture virus, but this was completely removed by heating at 56° for ½ hr.

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AN INVESTIGATION INTO THE PURIFYING EFFECT OF A FLUOROCARBON ON VACCINIA VIRUS

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Two main problems have been largely responsible for hampering work on the chemical composition of animal viruses.

Firstly, there is the difficulty of separating the virus particles from host cell constituents liberated along with them at the time of cell disruption. Although some animal viruses, such as influenza or vaccinia, have properties which make them relatively easier to purify than others (Ada and Perry, 1954 ; Craigie, 1932 ; Hoagland, Smadel and Rivers, 1940), members of the animal virus group as a whole are infinitely harder to obtain pure than, for example, certain insect viruses (Smith and Williams, 1958) and plant viruses (Steere, 1956).

The second problem is an extension of the first ; it consists, as has been pointed out elsewhere (Burnet, 1955), in the difficulty of establishing a criterion of purity once preparations of virus particles have been obtained. As Burnet (1955) has stressed, it is the absence of an adequate criterion on this crucial point which has rendered suspect so many investigations on the composition of animal viruses.

With regard to the first of these two problems, it has been claimed by Gessler, Bender and Parkinson (1956) that viruses can be purified and separated from host cell constituents if extracted from infected tissue by homogenization in a water-fluorocarbon mixture. The work reported did not include any adequate biological, morphological or other tests for the purity of the virus preparations which were made, but, as has been mentioned above, the difficulties in the way of applying satisfactory checks of purity form a major obstacle in this field. However, it has been shown quite recently in complement fixation experiments (Hummeler and Hamparian, 1957 ; Hamparian, Müller and Hummeler, 1958) that treatment of various animal virus suspensions with fluorocarbon did in fact effectively remove host antigens and anti-complementary activity, so that fluorocarbon might indeed be of value for the purification of such viruses.

Concerning criteria for assessing the purity of virus material, a new approach is suggested by some recent investigations of Palade and Siekevitz (1956*a* and 1956*b*). These workers have shown in an elegant series of studies that ultra-thin sectioning techniques for electron microscopy can be applied to pellets of cell fractions obtained by differential centrifugation. This provides a means of judging the homogeneity and the purity, morphologically, of the pellets down to the level of fine structure which can be resolved with the electron misroscope. Although such a check cannot yield information regarding the adsorption or loss of soluble substances by structures visible in a pellet, at least direct observations

on the morphological homogeneity of these structures provide a criterion of purity far superior to any hitherto available and one which can be applied to virus pellets just as readily as to those composed of cell fractions.

In view of the success obtained by Hummeler and Hamparian (1957) with fluorocarbon in their serological work, it was considered that an investigation of the virus purifying effect of a compound of this type would be valuable. Vaccinia virus was selected for this since it had been used in the original experiments with fluorocarbon (Gesslers *et al.*, 1956) and had the advantage of being relatively large and relatively easy to handle.

Preparations of the virus which had been treated with fluorocarbon have been subjected to high speed centrifugation in order to obtain pellets of the formed elements present. The pellets were fixed, embedded and sectioned for electron microscopy so that their purity might be checked by direct observation in the way introduced by Palade and Siekevitz (1956*a* and 1956*b*). Permanganate fixation (Luft, 1956) has been used throughout since preliminary investigations had shown that this revealed much unsuspected fine structure in the mature virus particles (Epstein, 1958*a*) and, in marked contrast to osmium-sucrose fixation (Palade, 1952 ; Caulfield, 1957), gave excellent preservation right through all samples of the pellets. In combination with the morphological work, checks have been made on the biological activity of the virus materials at each stage.

The present communication reports the results which have been obtained ; the findings of parallel cytochemical studies on the pellets of fluorocarbon-purified vaccinia virus are presented elsewhere (Holt and Epstein, 1958).

MATERIALS AND METHODS

Virus strain.—A Lister Institute strain of vaccinia virus was used ; it was obtained in 1954, through the kindness of Dr. D. McClean, as pulp from the 8th mouse skin passage. The virus was subsequently passed 11 times in the cells of mouse ascites Sarcoma 37 *in vivo* and 6 times on the chorio-allantoic membrane of chick embryos ; between some of the passages the virus was stored at −70° in sealed ampoules.

Eggs.—Fertile chickens' eggs were obtained throughout from the same source ; they were used after the embryos had been incubated for 10 to 11 days at 37°.

Suspending fluid.—The suspending fluid used for preparing the stock virus pool and in the titrations, consisted of 5 per cent inactivated rabbit serum in 0·01 M phosphate buffer at pH 7·38, brought to isotonicity by the addition of NaCl (0·792 g. per 100 ml.). The serum was inactivated by heating it to 56° for 30 min. shortly before use.

Stock virus pool.—Stock virus was prepared by grinding 5 infected chorio-allantoic membranes with sand and placing the ground-up material in 10 ml. of suspending fluid. The suspension was centrifuged at 300 *g* for 3 min. and the resulting supernatant fluid was kept as the virus pool ; it was stored in sealed hard glass ampoules in 1 ml. volumes at −70° until required.

Preparation of fluorocarbon-treated virus suspensions

For each experiment, the thawed contents of an ampoule of stock virus were diluted in serial ten-fold steps with suspending fluid and 0·1 ml. samples of the 10^{-4} dilution were inoculated on to the chorio-allantoic membranes of a group of eggs using false air sacs prepared by the standard technique (Beveridge and Burnet, 1946) ; preliminary titrations of the stock virus pool had shown that this dilution gave maximal confluent lesions on the chorio-allantois after incubating the inoculated embryos for 72 hr. at 37°.

About 5 chorio-allantoic membranes with maximal confluent lesions following inoculation and incubation in the manner just described, were harvested. The membranes, which weighed about 3 g., were placed with 15 ml. of Earle's balanced saline solution (Earle, 1943)

and 7·5 ml. of Arcton 63 (CF_2Cl-CCl_2F of Imperial Chemical Industries Ltd., London) in the 100 ml. vortex beaker of a Nelco homogenizer (Type N.I.R.D. supplied by Measuring and Scientific Instruments Ltd., London). The homogenizer was run at top speed (14,000 r.p.m.) for 75 sec. and the homogenate was then decanted and centrifuged at 300 *g* for 1 min. causing the Arcton layer to separate out at the bottom with the aqueous layer above, sometimes topped by a thin covering of frothy scum; this method of homogenization and centrifuging was followed throughout the preparation procedure. The aqueous layer was pipetted off and kept, whilst the Arcton layer was homogenized again with 10 ml. fresh Earle's solution and 5 ml. fresh Arcton; after centrifugation the aqueous layer from this second treatment was pipetted off and kept. A third cycle of homogenization and centrifugation was next done on the pooled aqueous layers from the first and second runs mixed with 10 ml. fresh Arcton. This treatment was repeated a fourth and fifth time, the aqueous layer from each run being homogenized with 10 ml. fresh Arcton in the next run. After the fifth treatment the Arcton layer was dead white in colour and the aqueous layer was clear, except for a slight milky opalescence; this final aqueous layer constituted the Arcton-treated virus suspension.

Preparation of pellets for electron microscopy from virus suspensions

About 10 ml. of virus suspension was centrifuged in the No. 40 (angle) rotor of a Spinco Model L preparative ultra-centrifuge (of Beckman Instruments Inc., Palo Alto, California) at an average of 105,000 *g* for 1 hr., the centrifuge being brought to a halt by means of the brake.

Fixation.—The supernatant was then decanted from the centrifuge tube, the tube was cut in half down its long axis and the pellet it contained was cut up in the open tube into sample cubes measuring up to 0·5 mm.; this sampling was done under a binocular dissecting microscope (Lomag of R. and J. Beck, Ltd., London, magnifying × 6·6) using two Hudson-type lachrymal sac knives. The samples were immediately transferred with one of the knives into a fixing bottle containing iced 0·6 per cent $KMnO_4$ buffered at pH 7·6 (Luft, 1956) and fixation was continued at 2° for about 1 hr.

Dehydration and embedding.—When fixation was completed the fixative was replaced by iced 30 per cent alcohol which was kept at 2° for 15 min. and then by a further change of iced 30 per cent alcohol which was allowed to warm to room temperature during the next 15 min., as recommended by Luft (1956). The samples of pellet were further treated by passage through 50 per cent alcohol, 70 per cent alcohol, 90 per cent alcohol, three changes of 100 per cent alcohol, a mixture of equal parts of absolute alcohol and catalysed *n*-butyl methacrylate and then 3 changes of catalysed *n*-butyl methacrylate; each change lasted 25 min. The methacrylate was catalysed by the addition of 1 per cent by weight of benzoyl peroxide. For embedding, the samples were placed in No. 00 gelatin capsules containing catalysed *n*-butyl methacrylate which had been prepolymerized for about 1 hr. at 57°. Polymerization was carried out at 58° for 14–18 hr.

Microtomy.—Sections were cut with a diamond knife (Fernández-Morán, 1956)—kindly presented to us by Professor Humberto Fernández-Morán—fitted with a holder (Epstein, 1958*b*) which made it suitable for use in a microtome designed for glass knives (Porter and Blum, 1953). The sections were flattened on 20 per cent aqueous acetone by the method of Sotelo (1957); those showing golden interference colours were selected for surveying the general arrangement of the pellets, whilst those showing silver interference colours were used to study the fine structure of the individual objects present. For examination, the sections were mounted on formvar coated copper grids without removal of the embedding medium.

Methods used in assay of virus

For the assay of virus in a given material, the following procedures were used:

Taking of samples.—Samples of fluids were taken with tuberculin syringes and were kept at 4°. Samples of the pellets were taken in the way described above for those used for electron microscopy; the samples were transferred with a lachrymal sac knife to a mortar and were vigorously ground with a pestle in a few drops of suspending fluid. After the grinding, the volume of the suspending fluid was made up to 10 ml. and a portion of this was taken in a syringe and kept at 4°. Each sample in an experiment was kept stored only until all other samples were ready for use.

Inoculations.—Inoculations were made on to the chorio-allantoic membranes of eggs using false air sacs made by a method similar to that described by Scott (1948). The inoculum consisted of 0·1 ml. and either 4 eggs (Expt. 4) or 5 eggs (Expt. 5 and 6) were used for each dilution of a sample. After inoculation, the eggs were incubated for 72 hr. at 37° and were then opened ; the inoculated membranes of all live embryos were removed, floated in saline in Petri dishes and the presence or absence of pocks was recorded.

General considerations

The biological parts of the experiments were done with strict aseptic technique ; tests for the presence of contaminating bacteria were negative.

For the Arcton treatment of the virus suspensions the apparatus and fluids were cooled before use to about 2° and homogenization was carried out with the vortex beakers surrounded by a bath of melting ice.

During the cycles of homogenization, clean chilled beakers were brought into use for the first, fourth and fifth runs so that the clinging froth of one run was not carried over on the beaker wall to the next, after the homogenate had been decanted for centrifugation.With the particular quantities of the fluids used it was important to adjust the position of each vortex beaker so as to leave the least possible clearance space between its base and the homogenizer blades.

For the preparation of pellets from the virus suspensions the Spinco centrifuge rotor was cooled before use to about 2° and the centrifuge refrigerator was switched on when centrifugation was started. This ensured that the material in the centrifuge did not warm up beyond 4° during the run.

Where titrations were performed, the inoculations were completed about 2½ hr. after harvesting the chorio-allantoic membranes from which the samples under test had been prepared. The different samples and their dilutions were dealt with in quick succession and material of a type which preliminary experiments had shown to be likely to have little biological activity (*e.g.*, supernatant fluid above a pellet) was inoculated before that likely to have greater activity (*e.g.*, virus suspension).

Electron microscopy was done with a Philips EM-100 fitted with a high resolution objective lens ; maximum image contrast was obtained by the use of a 30 μ objective aperture and a flat Wehnelt cylinder. Electron micrographs were taken using an accelerating voltage of 60 kV.

Experimental procedure

Experiments 1 to 3.—In each of these experiments a pellet was made from a fluorocarbon treated virus suspension ; samples were then taken from all areas of the pellet and were prepared for electron microscopy. The samples were investigated with the electron microscope by examining sets of serial sections cut from them at various levels.

Experiments 4 to 6.—In these further experiments, pellets were prepared and treated as before ; in addition, during this procedure samples were taken from the virus suspension, the supernatant fluid above the pellet (Spinco Supernatant) and from the fluids containing re-suspended fragments cut from different regions of the pellets. These samples were diluted in serial ten-fold steps with suspending fluid and portions of the various Dilutions were used for egg inoculation.

Calculation of results

From the number of chorio-allantoic membranes which developed pocks following the inoculation of each dilution of a sample (table), that dilution of the sample which would have caused lesions in 50 per cent of chorio-allantoic membranes when given in a dose of 1 ml., was calculated. The method of calculation used was that of Reed and Muench (1938) and the results have been recorded in the table under the heading of Lesion dilution 50 (LD_{50}).

RESULTS

Macroscopic appearance of the pellets

The pellets measured about 4 mm. in diameter and had a characteristic appearance (Fig. 1). This consisted of a firm, dense white, round zone 1 to 2

mm. across, which lay in the upper central part of a pale grey ring; the ring occupied most of the outer portion of the pellet and there was a greyish tongue protruding into its centre from the lower edge of the white zone (Fig. 1). These regions merged into, and were covered by, transparent, moderately firm, gelatinous material which formed the upper layers of the pellet.

Electron microscopy of the pellets

Dense white zone.—The dense white zone consisted entirely of uniform particles measuring about 300 mμ by 200 mμ (Fig. 2); no other formed elements were

EXPLANATION OF PLATES

All the figures, apart from the first one, are electron micrographs of various regions of pellets prepared from fluorocarbon-treated vaccinia virus suspensipns.

FIG. 1.—View of the lower part of a centrifuge tube showing a pellet prepared by subjecting a fluorocarbon-treated vaccinia virus suspension to an average force of 105,000 *g* for 1 hr. The pellet consists of a dense white zone lying in the upper central part of a pale grey ring. The ring occupies the outer portion of the pellet and there is a greyish tongue protruding into its centre from the lower edge of the white zone. Light reflections can be seen at the base of the tube and on the right of the pellet. × 6.

FIG. 2.—Survey picture of a section cut through a representative region of the dense white zone of a typical pellet. Randomly orientated uniform particles measuring about 300 mμ by 200 mμ are the only formed structures present. × 13,000.

FIG. 3.—Small area of a section cut through the dense white zone of a pellet showing its composition in greater detail. Varying amounts of the individual randomly orientated particles are present in the section; those which have been cut tangentially have only a thin surface sliver included, in contrast to the more substantial segment taken from those cut nearer their centres. × 40,000.

FIG. 4 to 7.—Individual mature vaccinia virus particles from sections of the dense white zones of various pellets; the particles have been cut in different planes. × 200,000.

FIG. 4.—Longitudinal section across the shortest dimension of a particle showing the slight flattening. The outer limiting membrane consists of two electron-dense layers 5 mμ apart separated by a less dense layer in between. This limiting membrane covers a narrow zone of slight electron-density (*oz*), except at the centre of each of the two widest surfaces of the particle where an electron-dense lateral body (*db*) is interposed. The narrow zone of slight electron-density is limited centrally by another membrane which in turn surrounds an inner body or nucleoid. The nucleoid itself is differentiated into a central electron-dense area with an area of less density around it.

FIG. 5.—Longitudinal section across the widest dimension of a particle; this is at right angles to the plane of the section shown in Fig. 4, and along the line A–B. The layers of the outer limiting membranes are well seen as is the discoidal shape of the central electron-dense area of the nucleoid.

FIG. 6.—Transverse slightly oblique section through a particle along the line C–D of Fig. 4. The electron-dense lateral bodies (*db*) can be seen under the limiting membrane, separated from the nucleoidal membrane by the narrow zone of slight electron-density.

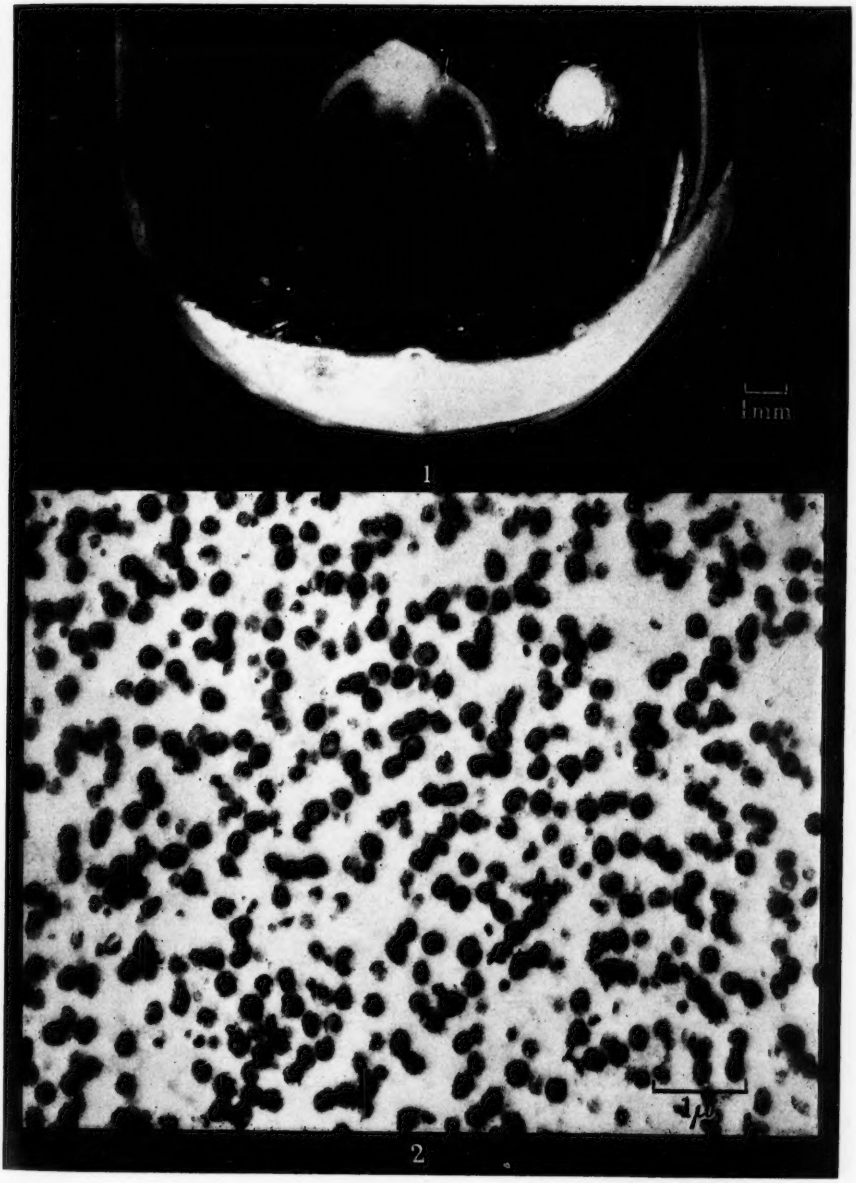
FIG. 7.—Section through a particle in almost the same plane as in Fig. 4; there is some asymmetry present and only one lateral body (*db*) has been cut through. The three-layered structure of the nucleoidal membrane is particularly clear.

FIG. 8.—Section through the gelatinous region of a pellet. × 40,000.

FIG. 9.—Detail of a small area of a section through the gelatinous region of a pellet. The granular appearance can be seen to be due to the presence of fine beaded threads. × 100,000.

FIG. 10.—Section through the area of a pellet where the dense white zone ends and the overlying jelly begins. The particles of the dense white zone are present on the right of the field with the sharply demarcated jelly covering this zone, on the left. × 30,000.

FIG. 11.—Survey picture of a section through the ring region of a pellet. The fuzzy jelly is arranged in strands forming a coarse meshwork. These are packed down into dense laminae in the lower layers of the pellet shown at the bottom of the field. × 6,000.



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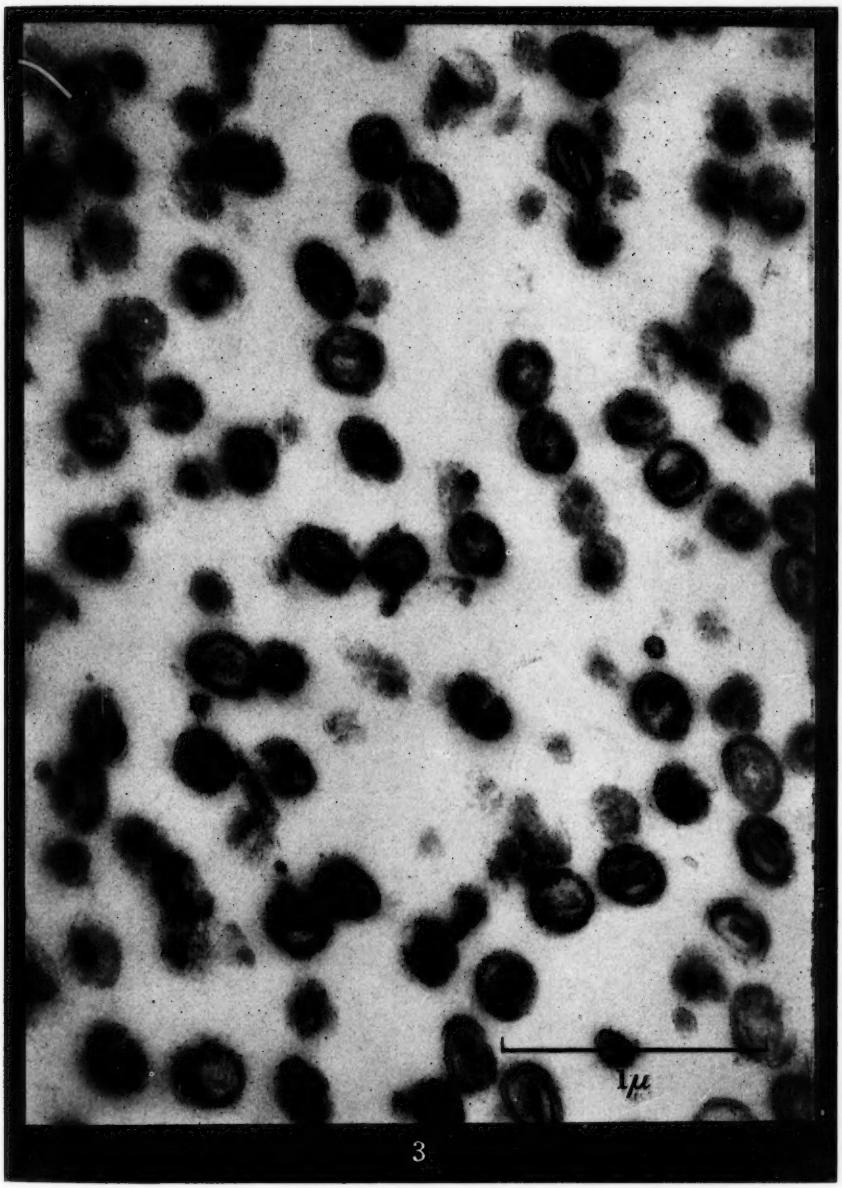

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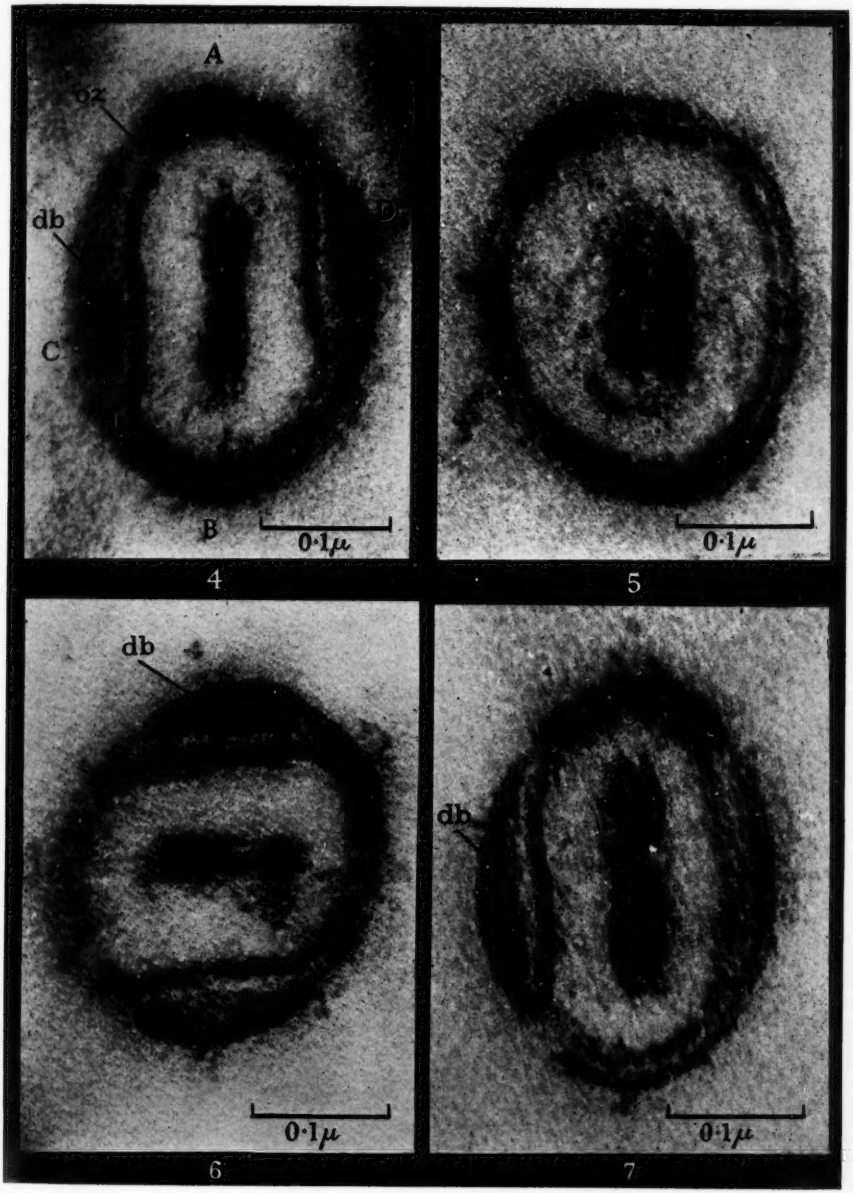

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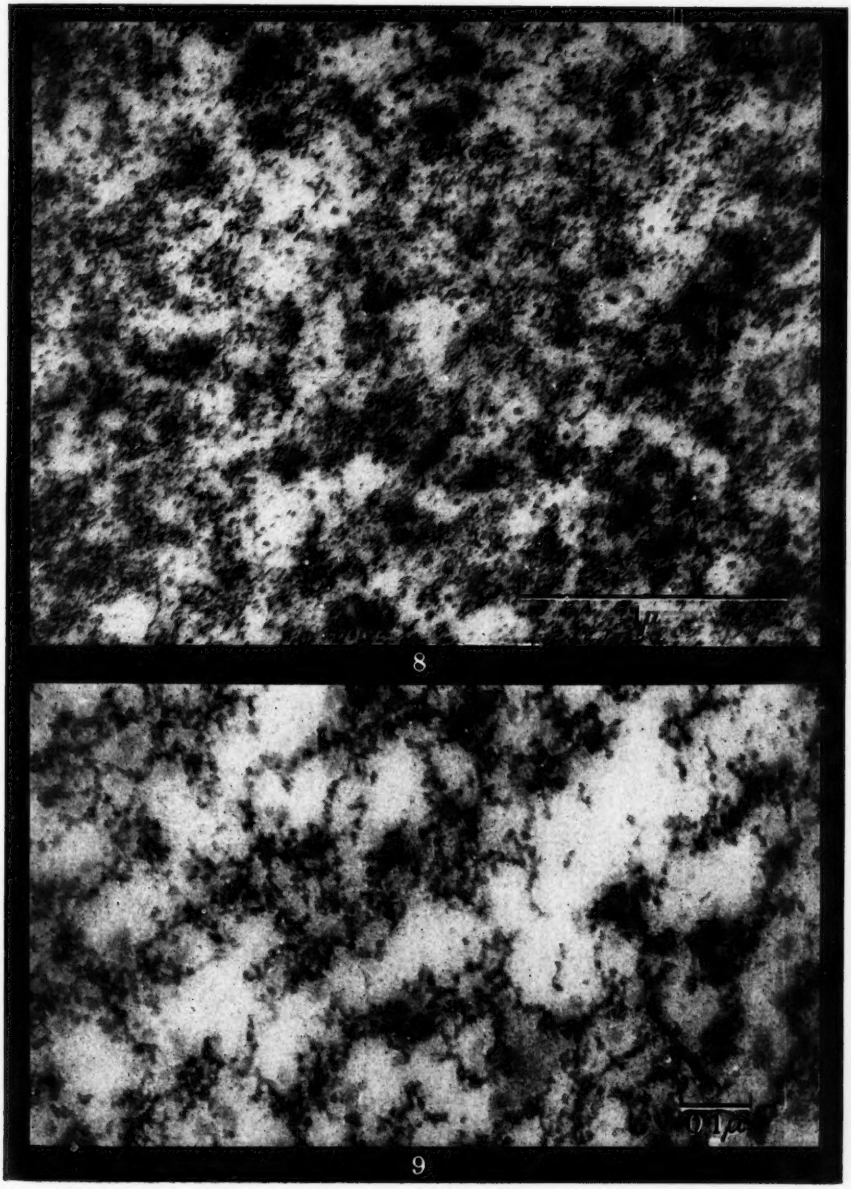

8

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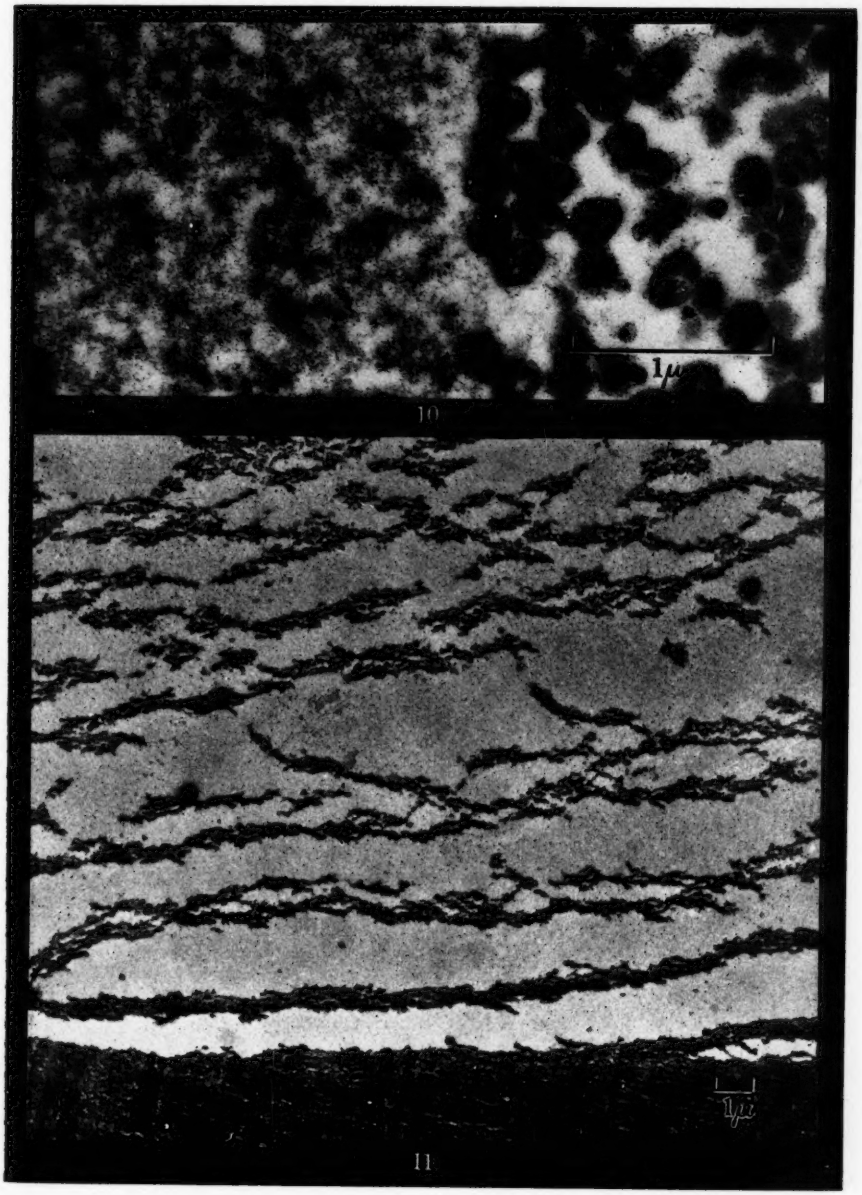
Epstein.



Epstein.

observed. The particles were orientated at random and varying amounts of individual particles were present in a given section ; those particles cut tangentially had only a thin surface sliver included, in contrast to the more substantial segment taken from those cut nearer their centres (Fig. 3).

In shape, the particles resembled short, slightly flattened bricks with markedly rounded edges and ends (Fig. 2 to 7). Their fine structure consisted of an outer double limiting membrane whose two electron-dense layers lay about 5 mμ apart with a less dense layer in between (Fig. 4 to 7). This limiting membrane covered a narrow zone of slight electron density (Fig. 4 to 7) except at the centre of each of the two widest surfaces of the particles where an electron-dense lateral body was interposed (Fig. 4, 6 and 7). The narrow zone of slight electron-density was limited centrally by a further double membrane enclosing an inner area or nucleoid (Fig. 4 to 7) ; the double membrane of the nucleoid also had two electron-dense layers about 5 mμ apart separated by a less dense layer (Fig. 7). The nucleoid itself was differentiated into a central disc-shaped electron-dense region surrounded by a region whose slight electron-density resembled that of the zone lying immediately below the outer limiting membrane (Fig. 4 to 7).

Jelly, ring and tongue.—The jelly which formed the upper strata of the pellet consisted of uniform, fuzzy material (Fig. 8, 10 and 11) which had a granular appearance at higher magnification owing to the presence of fine beaded threads (Fig. 9). In areas of the pellet immediately surrounding the dense white zone, particles identical with those composing this zone were found scattered in the jelly ; the jelly lying above the dense white zone was, however, sharply demarcated from the underlying particles (Fig. 10). Above the ring and central tongue the jelly was arranged in strands forming a coarse mesh-work ; this was packed down into dense laminae in the lower layers of these regions (Fig. 11), the dense packing coinciding with the greyish opacities visible macroscopically as the ring and tongue (Fig. 1).

Combined electron microscopy and titrations

The results shown in the Table are of the 3 experiments in which the biological activity of the virus was followed during the preparation of pellets and was related to the morphological observations made with the electron microscope on samples from different regions of each pellet.

It can be seen that the infectivity of each virus suspension was very considerably greater than that of the supernatant fluid (Spinco Supernatant) above the pellet formed by high speed centrifugation of the suspension (table). After this centrifugation something of the order of 99·9 per cent of the biological activity was eliminated from the virus suspension on 2 occasions (Expt. 4 and 6) and much more than this on another (Expt. 5). That this biological activity had been deposited in the white zone of the pellets is shown by the fact that in each case a very small sample from this zone was highly active (Expt. 4, 5 and 6) even when re-suspended in as great a volume of fluid (10 ml.) as the original virus suspension from which the whole pellet came. In contrast, similarly re-suspended small samples of jelly from the centre of the pellets (Expt. 4 and 5), or from the edge (Expt. 6), possessed relatively little activity.

Samples taken from the white zones of the pellets prepared in Expt. 4, 5 and 6 were seen, when examined in the electron microscope, to be composed entirely of uniform particles having the characteristic shape and fine structure described

TABLE.—*The Biological Activity of Samples Taken at Different Stages During the Preparation of Pellets from Fluorocarbon Purified Virus Suspensions (Titration on Eggs C.A.M.)*

Expt. No.	Sample	Dilutions of sample and result: Denominator—number of live eggs harvested; Numerator—number of live eggs with lesions									LD_{50} per ml. of sample
		10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
4	Virus suspension	—	—	—	—	4/4	4/4	3/4	0/4	0/4	$10^{-7.3}$
	Spinco supernatant	—	—	4/4	2/4	0/4	0/4	0/4	0/4	0/4	10^{-4}
	White pellet	—	—	—	3/3	4/4	2/4	0/4	0/4	0/4	10^{-6}
	Central pellet jelly	—	3/3	4/4	0/4	0/4	—	—	—	—	$10^{-3.5}$
5	Virus suspension	—	—	—	—	—	5/5	5/5	1/5	0/5	$10^{-7.6}$
	Spinco supernatant	—	—	5/5	1/5	0/4	0/4	—	—	—	$10^{-3.6}$
	White pellet	—	—	—	—	5/5	4/4	0/4	0/5	—	$10^{-6.5}$
	Central pellet jelly	—	—	5/5	3/5	0/5	0/4	—	—	—	$10^{-4.1}$
6	Virus suspension	—	—	—	—	—	4/4	4/4	1/5	0/5	$10^{-7.6}$
	Spinco supernatant	—	5/5	5/5	5/5	2/4	0/5	—	—	—	10^{-5}
	White pellet	—	—	—	—	5/5	3/4	0/5	0/3	—	$10^{-6.3}$
	Peripheral pellet jelly	5/5	4/5	0/5	0/4	0/5	—	—	—	—	$10^{-2.4}$

above (Fig. 2 to 7). Samples of the gelatinous areas of the pellets were found to be free of particles (Fig. 8, 9 and 11) when examined in this way.

DISCUSSION

The technique of fluorocarbon purification used in the present investigation is based on that described by Gessler *et al.* (1956) in their original work with compounds of this type. These workers used a mixture of fluorocarbon and *n*-heptane but noted that if Genetron 226 were the fluorocarbon chosen, *n*-heptane was not required. Genetron 226 is the trade name used in the United States for the fluorocarbon $CF_2Cl\text{-}CCl_2F$ which is sold as Arcton 63 in Great Britain. It was because of its suitability for use without the possible complicating factor of *n*-heptane that Arcton 63 was selected for the present experiments. Although 3 applications of fluorocarbon were suggested by Gessler and his colleagues (Gessler *et al.*, 1956), trials carried out in preparation for the work reported here showed that it was only the Arcton of the fourth or fifth application that became white in colour and free of material; 5 treatments with Arcton were therefore adopted as a standard procedure.

The fixative used here in preparing samples of pellets for electron microscopy was likewise chosen as a result of preliminary tests. Although buffered osmium (Palade, 1952) has been widely proved to be an outstandingly successful fixative for electron microscopy, particularly if sucrose is added to it (Caulfield, 1957), when applied to samples from the white zones of pellets prepared from purified vaccinia virus suspensions the results were disappointing. Only a narrow outer layer of the sample contained recognizable particles and not all of these were well preserved; permanganate fixation (Luft, 1956) on the other hand, gave excellent preservation of the particles right through the samples.

In the titration experiments, care was taken to ensure that the samples of material whose biological activity was to be compared were kept at a standard temperature and in the same suspending fluid until all were ready for serial dilution

and inoculation. The results obtained were further validated by inoculating the biologically feebler materials before the more active.

From the results which have been obtained it is evident that if vaccinia virus grown on the chick chorio-allantois is treated with fluorocarbon 5 times in a way similar to that suggested by Gessler *et al.* (1956), a suspension can be obtained which is entirely free of formed host cell constituents. This is shown by the fact that the pellets made by high speed centrifugation of such suspensions consisted only of regular, formed particles (Fig. 2 and 3) together with fuzzy gelatinous material (Fig. 8, 9, 10 and 11). The particles were of a size appropriate for vaccinia virus and their structure was uniform (Fig. 2 to 7). In general internal arrangement they closely resembled osmium-fixed mature extra-cellular vaccinia virus (Morgan, Rose, Ellison and Moore, 1954; Peters, 1956) and the results of the titration experiments (table) clearly show that the biological activity of the pellets lay in the region composed of the particles. It must be borne in mind, however, in considering the activity shown by samples from different areas of the pellets that the results obtained are not strictly quantitatively comparable, since it was not possible to cut out fragments of an exact standard weight or volume. Nevertheless, the samples were all of the same order of size whilst the differences of biological activity ranged from a factor of about 500 (Expt. 4 and 5) to a factor of 10,000 (Expt. 6). Furthermore, these results probably give an underestimate of the biological activity residing in the particles forming the dense white zones of the pellets, since in re-suspending the samples from these zones it is more than likely that the packed particles were dispersed in clumps or aggregates rather than singly.

Thus, the fluorocarbon treatment yielded suspensions in which the only formed structures were virus particles, but which contained at the same time the fuzzy material deposited by high speed centrifugation as the gelatinous portion of the pellets. That the morphological criterion of purity applied to the virus preparations was able to detect the gelatinous material says much for the method of Palade and Siekevitz (1956*a* and 1956*b*) which was used.

The presence of the gelatinous material makes it impossible to use pellets from fluorocarbon-treated vaccinia virus suspensions for chemical analysis of the virus, since the jelly, whatever its relevance, represents a contaminant. In fact, the jelly is of very great importance in this context because cytochemical studies (Holt and Epstein, 1958) have shown that it contained a substantial amount of free deoxyribonucleic acid. It is of interest to note in this connection that egg-grown Rous virus treated with fluorocarbon has been isolated with an apparently similar jelly (Epstein, 1958*c*) which likewise contained much free deoxyribonucleic acid (Epstein and Holt, 1958)

The jelly may also be of importance from a serological point of view, for in the experiments of Hamparian *et al.* (1958) viral antigens prepared from chick chorio-allantoic membranes were found to contain 2 types of reactive host components. One of these could be removed by a single treatment with fluorocarbon, whereas the other was still present in quantity even after 5 such treatments. It might be that this latter type of host component corresponds to some constituent of the jelly found in the present investigation.

The morphological observations on the mature vaccinia virus particles reported here include certain findings of interest. Thus, although the permanganate fixed particles showed a general internal arrangement similar to that described

in osmium-fixed mature extra-cellular vaccinia virus (Morgan *et al.*, 1954 ; Peters, 1956), they differed in their fine structure in some important details. Firstly, the particles always appeared plumper in the sections than those fixed with osmium (Peters, 1956) ; this was particularly evident in particles sectioned longitudinally across their shortest dimension (Fig. 4). The plumpness was probably emphasized by the use of Sotelo's (1957) technique for ensuring complete flattening and expansion of the sections, but even in preliminary work where this was not done (Epstein, 1958*a*) the particles observed were plumper and less flattened than those fixed by osmium. Secondly, the outer zone of the virus between the outer limiting membrane and the membrane surrounding the nucleoid, was narrower and less electron-dense than in osmium-fixed material. The last and most important difference was the striking differentiation of the nucleoid into an inner region of great electron-density and an outer region of slight electron-density (Fig. 4 to 7). The presence of these two hitherto unsuspected structurally distinct parts in the nucleoid is of special significance in the light of earlier enzymatic digestion studies on viruses of the pox group. For, the fact that in pepsin-treated particles the nucleoid cannot be hydrolized by desoxyribonuclease alone, but only by desoxyribonuclease followed by a further pepsin digestion, has been taken as an indication that the nucleoid contains protein as well as nucleic acid (Peters and Stoekenius, 1954). It might be that the two distinct regions found in the nucleoid of the permanganate-fixed particles are linked in some way to the presence of these two nucleoidal substances.

Although the presence of the jelly in the pellets shows that fluorocarbon treatment does not yield a vaccinia preparation containing only virus particles as had been hoped, the findings of the present work are nevertheless encouraging for a number of reasons.

Firstly, the virus has in fact been obtained so free of contaminants in the firm white zones of the pellets that separating it from the substances composing the jelly which lay around these zones is considered a feasible undertaking.

Secondly, the particles in the white zones form an extremely valuable source of easily and rapidly prepared virus material for morphological study, as the present findings on the structure of the virus indicate. Furthermore, just as permanganate was applied with ease to the fragments cut from the pellets, so other fixatives and reagents can be similarly applied. This point is illustrated by work on the composition of the nucleoid of the Rous virus (Epstein and Holt, 1958), in which samples of fluorocarbon purified pellets of this virus were treated with various fixatives and specific enzymes.

Lastly, the fact that infectivity can be shown to depend entirely on the presence of particles free of formed host cells constituents, as has been done here in the combined biological and morphological experiments, offers a most useful method for identifying unknown particles with specific viruses. This procedure has been used to confirm the nature of the particles found in association with Rous tumours (Epstein, 1958*c*) and could equally well be applied in the case of other similar virus induced conditions both neoplastic and infectious.

SUMMARY

Experiments are described which were performed to investigate the purifying effect of a fluorocarbon (CF_2Cl-CCl_2F) on vaccinia virus. The virus was grown on the chorio-allantoic membranes of chick embryos and was separated from

host cell constituents at a water-fluorocarbon interface ; the virus suspensions were subjected to a force of 105,000 *g* for 1 hr. and the pellets obtained were fixed with permanganate, embedded and sectioned for electron microscopy. Direct observation of the sectioned pellets with the electron microscope has been used as a check on their purity. In combination with the morphological work, biological tests of virus activity have been made on the material at each stage in the experiments.

The techniques which have been used are described in detail.

The results show that fluorocarbon treatment enabled virus suspensions to be made which were entirely free from formed host cell constituents but which nevertheless contained other important host substances. This was demonstrated by the fact that the pellets made by high speed centrifugation of the suspensions consisted of a firm white zone composed entirely of regular brick-shaped particles measuring about 300 mμ × 200 mμ, as well as a gelatinous region having a fuzzy, granular fine structure. The biological tests have established the viral nature of the particles in the pellets. They have shown that about 99·9 per cent of the infectivity of the virus suspensions was eliminated by the gravitational force applied and that this activity came to reside, after the centrifugation, in that part of each pellet which was composed of the particles.

A description is given of the fine structure of permanganate-fixed mature vaccinia virus particles including an account of the structural differentiation found, for the time, in the nucleoid.

Both the limitations and the possible usefulness of fluorocarbon purification of an animal virus such as vaccinia, are discussed in the light of results obtained.

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SURVEY OF PAPERS

ELDER and WILHELM, testing saline dilutions of human serum in guinea-pig skin, have shown the presence of two permeability factors. Freshly harvested undiluted serum contains a factor IPF/Nat which is not inhibited by soya bean trypsin inhibitor (SBTI), guinea-pig serum inhibitor (IPF), or mepyramine. This factor is possibly a result of species incompatibility. The other factor, PF/Dil is detectable after dilution of the serum, and like guinea-pig PF/Dil, is inhibited by SBTI and guinea-pig IPF but not by mepyramine (p. 335).

MILL, ELDER, MILES and WILHELM describe the partial purification from human serum of a capillary permeability factor activable on dilution and its inhibitor. The factor differed from those of rat and guinea-pig serum in its relative instability and its association with β- rather than α-globulin (p. 343).

VAN DEN BRENK, continuing his study of the role of histamine in irradiation responses, found that the morphological changes in mast cells produced by irradiation differed from those due to Compound 48/80, and that irradiation skin damage developed earlier and was more severe in histamine-depleted rats. The response of irradiated skin to inflammatory stimuli was normal, and neither the response of the mast cells to Compound 48/80 nor the shock produced by it was modified by irradiation (p. 356).

SULITZEANU has investigated the relationship of the agar diffusion patterns to the agglutinating and protective properties of *Brucella suis* antisera. Using a variety of absorption and immunization procedures, antisera of widely different agar diffusion patterns were obtained; none of them could be correlated with the presence or absence of agglutinins. Good correlation existed between agglutinating and passive protection power of sera in mice. Two agglutinogens were demonstrated in insoluble cell debris (p. 367).

STOREY and VARASDI study the repair of fractures in the rat during the administration of aminoacetonitrile (AAN) and following its withdrawal. The left tibia of each rat was fractured and allowed to heal. The animals were subsequently killed and the fracture site removed for histological examination. During AAN administration an excessive formation of poorly formed giant callus was produced. When the administration of AAN ceased normal repair processes began with the laying down of collagen and bone formation. The mode of action of AAN is discussed and it appears to produce a disturbance in the formation of collagen and bone from the connective tissue ground substance (p. 376).

POPE and MURIEL F. STEVENS, extending their finding that crystalline diphtheria toxin-protein contains several antigens, have shown by an optical method that the apparent increase of L_f value produced by treatment of toxin-protein with K_3PO_4 is due to the presence of a phosphate stable antigen which gives a symmetrical flocculation curve. The flocculating properties of other antigens are inhibited but without complete loss of combining properties (p. 386).

ADAMS, FERNAND and SCHNEIDEN investigate the cytochemical changes in the liver and various glands of rodents maintained on a diet of cassava and determine the effects of dietary supplements of protein, amino acids and vitamins in modifying the changes observed. The rodents showed a loss of stainable protein in the glandular epithelium from many sites and these histological changes could not be reversed by dietary supplements of incomplete protein, individual amino acids, vitamins or lipotrophic factor: only complete protein was effective. The relationship of the syndrome found in cassava-fed animals to kwashiorkor is discussed and the authors conclude that the two conditions are essentially similar (p. 393).

DODSON has studied the effect of foetal ablation upon the blood pressure of hypertensive rats. He shows that the fall of blood pressure during the latter part of pregnancy is dependant upon the presence of the placenta and is independent of the presence of the foetus. He further shows that there is a parallel between the fall of blood pressure in hypertensive pregnant rats and the refractoriness to renin showed by normal rats during pregnancy (p. 405).

WENDEL, GRIFFIN and BROPHY determine the survival time and the biochemical changes occurring in rats subjected to a standardized bowel shock and evaluate the therapeutic effects of various agents. The authors produced strangulation obstruction in rats by slip-knot sutures placed round the circumference of the bowel in two places 5–7 cm. apart and occluded the mesenteric blood supply in a similar fashion. The occlusion was released after a period of 4 hr. and this resulted in a 95 per cent mortality. At post mortem numerous petechial haemorrhages were present in the lungs, thymus, liver and gastro-intestinal tract and investigation of the blood showed a marked rise in the blood ammonia concentration with a fall in the urea level. The administration of neomycin sulphate, Permutit T and chlorpromazine at a dosage of 20 mg./kg., prior to the strangulation obstruction procedure protected the animals against the lethal effects of shock. In these treated animals the blood ammonia was increased to almost the same levels as in the untreated animal, but the blood urea also showed an increase. Methionine was found to enhance the lethal effects of strangulation obstruction and neomycin sulphate was effective in protecting these animals (p. 411).

STEIN and WOLMAN describe the changes occurring during the process of wound healing in scorbutic guinea-pigs using histo-chemical techniques. In the healing area of scorbutic animals the authors demonstrate an increase of PAS-positive material which stains red with bi-col when compared with control animals. Twenty-four hours after the administration of ascorbic acid to scorbutic animals there followed a regression of the relative amounts of these stainable polysaccharides towards the state prevailing in the normal control animals and this change was accompanied by an enhanced formation of granulation tissue (p. 418).

PLOWRIGHT and FERRIS have grown a strain of sheep pox virus in monolayer cultures of sheep, goat and ox cells. The virus was more extensively cytopathogenic for testis than for kidney cultures and was passaged serially with good yields and no attenuation of virulence for sheep. Although neutralization was incomplete, it was possible to demonstrate and measure, by this method, antibodies in convalescent sheep sera (p. 424).

EPSTEIN has used the electron microscope as a means of assessing the degree of purification achieved by treating vaccinia virus with a fluorocarbon. The virus was grown in the chorioallantoic membrane of chick embryos, separated from the host cells at a fluorocarbon-water interface, and sedimented by ultra-centrifugation. The deposit thus obtained was embedded and sectioned for electron microscopy. The implications of the results obtained are discussed (p. 436).